The Evolution and Maintenance of Body Colour Polymorphism in *Bombus ruderatus* in the South Island, New Zealand

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Abstract

Explaining the wide range of animal colouration in the natural world is a key issue in evolutionary biology. Bumble bees are often brightly coloured and show a range of colours and colour patterns in different species as well as considerable variation within species. The large garden bumble bee, *Bombus ruderatus*, is highly variable in its degree of black (melanic) colouration, with morphs ranging from the familiar yellow and black bands (banded) through intermediate forms to morphs that are totally melanic. The aim of this research was to determine what might be maintaining the colour polymorphism in populations of *B. ruderatus* in the South Island, New Zealand. Colouration of worker bees was measured using a digital photography method and found to be significantly different across sample sites. To look at potential adaptive functions of body colour in *B. ruderatus*, three hypotheses of thermoregulation, desiccation tolerance and Müllerian mimicry were tested by comparing patterns of variation in melanism to patterns of variation in climatic variables (temperature, rainfall, humidity) and abundance of conspecifics. In order to address the possibility that selectively neutral processes were more important than selection, the genetic structure of *B. ruderatus* populations was characterised and compared to the pattern of variation in melanism. The colouration of individuals from the same population collected at different times in the season was compared to evaluate whether body colour was plastic and any support for the genetic basis of melanism in *B. ruderatus* was also assessed by determining any relationship between relatedness and degree of melanism. The results suggest that differences in the degree of melanism between populations are greater than the differences expected through selectively neutral forces alone and, therefore, that the pattern of variation in melanism is likely a result of selection and/or phenotypic plasticity in addition to gene flow and genetic drift. Although a global model consisting of four climatic variables and the abundance of conspecifics explained a small proportion of the variation in melanism, no support was found for any specific hypothesis relating to the adaptive function for body colour. Instead the results suggest that some combination of factors, most likely including factors not measured in this study, is influencing the frequency of melanic morphs. In addition, there was evidence that body colour was influenced by phenotypic plasticity and that melanism has a low heritability in *B. ruderatus.* Taken together, these results imply that patterns of melanism across *B. ruderatus* populations are complex and it is likely that multiple factors are influencing melanism in concert.

CHAPTER 1: General introduction

Colour variation in animals

Differences in animal colouration provide one of the most obvious examples of variation in nature and explaining why this variation exists is a key issue in evolutionary biology (Bennett & Théry 2007). A range of possible functions for colouration has been proposed and in many cases body colour is expected to influence the fitness of individuals (Protas & Patel 2008). These functions have mainly related to one of three hypotheses: colouration is involved in 1) the concealment of individuals, 2) communication/signalling (intra- and inter-specific) between individuals and 3) physiological processes (True 2003; Caro 2005; Protas & Patel 2008). Many examples of concealment from predators using camouflage or crypsis involve background matching of colour and colour patterns (Stevens & Merilaita 2009). Colour can be a component of signalling traits that influence mate choice involved in sexual selection (Andersson 1994; Griffith *et al.* 2006), such as bright pigmentation of bird plumage (Hill 1991; Badyaev & Hill 2000) or brightly coloured skin patches of some primates (Caro 2005). Intraspecific colour signals can also be used to indicate status in dominance and competitive interactions (Tibbetts & Lindsay 2008; Green & Field 2011). Warning colouration, or aposematism, is a form of interspecific signalling when prey has conspicuous, contrasting body colouration that advertises that it is unpalatable to potential predators (Endler & Mappes 2004; Mappes *et al.* 2005; Marples *et al.* 2005). Lastly, colouration can affect physiological processes that influence thermoregulation (True 2003; Caro 2005), pathogen resistance (True 2003; Bailey 2011; Jacquin *et al.* 2012; Dubovskiy *et al.* 2013), UV resistance (True 2003), energy storage (Ahsaei *et al.* 2013) and desiccation tolerance (True 2003; Rajpurohit *et al.* 2008).

Colour polymorphism is a specific case of colour variation defined as the presence of individuals within the same population of the same sex and age that display two or more genetically determined colour morphs (Roulin 2004; Gray & McKinnon 2007). This makes colour polymorphism interesting from an evolutionary perspective because directional selection should to lead to an increased frequency of the fittest morph. For example, the benefit of warning colouration increases with the frequency of individuals with similar signals and is expected to promote monomorphism, that is a single colour dominating (Endler & Mappes 2004; Mappes *et al.* 2005). The same principle applies to Müllerian mimicry (Müller 1879), although in this case, two or more different species are expected to converge on the same colour morph. However, in colour polymorphic populations the

rarest morphs are too frequent to be the outcome of recurring mutation alone (Gray & McKinnon 2007).

Multiple mechanisms have been put forward as possible ways in which colour polymorphisms can be maintained including several within population processes such as apostatic (frequency dependent) selection (Galeotti *et al.* 2003; Svensson & Abbott 2005; Bond 2007), disruptive selection (Galeotti *et al.* 2003; Galeotti & Rubolini 2004; Jacquin *et al.* 2012), mutation and selection balance (Roulin 2004) and heterosis (heterozygote advantage) (Gratten *et al.* 2012). In addition to purely adaptive hypotheses, selectively neutral processes such as genetic drift (O'Hara 2005; Oxford 2005; Hoffman *et al.* 2006; Sánchez-Guillén *et al.* 2011) and gene flow (*i.e.* selection and migration balance / hybridization between populations) (Roulin 2004; Rosenblum 2006; Gray & McKinnon 2007; Hedrick & Ritland 2012) may play a role in the maintenance of colour polymorphism when acting in conjunction with selection (Gray & McKinnon 2007). Variation in body colour could alternatively be the outcome of phenotypic plasticity (Protas & Patel 2008). An individual's phenotype is the result of a series of developmental processes that are shaped by both genes and the environment; as such the same genotype can result in multiple different phenotypes when development occurs under different environmental conditions (West-Eberhard 1989; Nijhout 1999; Nijhout 2003).

A form of colouration that has been the focus of much research is melanism. Melanism is the occurrence of dark or black colouration and is common in many animal groups (Majerus 1998; True 2003). Selective mechanisms acting upon dark body colour can be summarised as either visual or non-visual. Visual mechanisms relate to the concealment and signalling hypotheses, including aposematism, crypsis and mimicry (True 2003). Non-visual mechanisms relate to physiological processes, including thermoregulation, desiccation tolerance and UV resistance (True 2003).

The classic model of a visual selective mechanism acting on melanism is the case of crypsis in the peppered moth, *Biston betularia.* The frequency of the melanic form (f. *carbonaria*) of this species increased in urban areas of England in the 19th century whilst remaining relatively uncommon in rural areas (Majerus 1998; Cook 2003). The effect of industrialisation and the pollutants this caused changed the environment in urban areas so that trees lost lichen growth and many surfaces became darker. This in turn gave melanic morphs a selective advantage, as they were now better camouflaged from avian predators than non-melanics when resting in urban environments (Majerus 1998; Cook 2003). Frequencies of *carbonaria* began to decline in urban areas in the 1970s as the change from coal to oil and gas in the 1960s, smoke abatement legislation and rebuilding in some

urban areas began to reduce and reverse the impact of pollutants (Majerus 1998; Cook 2003). More recent work found that the cline of morph frequencies in 2002 was still present although much shallower and high levels of gene flow between urban and rural areas are able to maintain moderate frequencies of *carbonaria* in intermediate rural areas even when selection against melanic individuals is strong (Saccheri *et al.* 2008).

Non-visual mechanisms selecting for melanism can also be important for insects (True 2003). One of the most commonly investigated non-visual mechanisms is thermal melanism. The thermal melanism hypothesis suggests that ectotherms will benefit from dark colouration in colder environments due to faster rates of heating and cooling, with higher body temperatures at the same levels of solar radiation (True 2003; Clusella-Trullas *et al.* 2007). Melanism has been linked to increased flight activity and egg maturation rates at colder temperatures in *Colias* butterflies (Ellers & Boggs 2004). Karl *et al.* (2009) found that variation in wing and pupal melanism in two copper butterfly species (*Lycaena hippothoe* and *L. tityrus*) followed an altitudinal cline, finding increasing melanism with increasing altitude. In a review of thermal melanism, Clusella-Trullas *et al.* (2007) concluded that in most cases darker coloured individuals are more common in the cooler parts of their range and in general darker morphs have higher fitness in colder environments.

As there is a range of potential functions for melanism, it is probable that variation in melanism will be subject to both visual and non-visual selective mechanisms simultaneously (True 2003). The two-spot ladybird *Adalia bipunctata* and the ten-spot ladybird *A. decempunctata* are both aposematic species that are polymorphic with red and black colouration. Brakefield (1985) found that in the Netherlands, frequencies of different colour morphs of both ladybird species group geographically, suggesting that they form Müllerian mimicry rings. He also found variation in the frequency of melanic morphs, which was negatively correlated with sunshine hours in *A. bipunctata,* but not in *A. decempunctata*. The difference appeared to be linked to habitat use that results in *A. bipunctata* being more exposed to sunshine than *A. decempunctata* (Brakefield 1985). As in the peppered moth, frequencies of melanic individuals were also correlated with levels of pollution in the UK, which appeared to be linked to the effect of smoke causing reduced sunshine (Brakefield & Lees 1987). The thermal properties of melanic and non-melanic varieties of *A. bipunctata* differ, due to differences in the absorption of solar radiation, with melanic forms heating and cooling faster (Brakefield & Willmer 1985, de Jong *et al.* 1996). A re-evaluation of the cline in the Netherlands by de Jong & Brakefield (1998) found that changes in the frequencies of melanic morphs were consistent with those expected over a cline of climatic conditions with respect to the thermal melanism hypothesis.

These studies highlight the likelihood that multiple selective mechanisms, both visual and non-visual, will act upon variation of melanism in insects (True 2003; Forsman 2011) and insect body colour in general (Ibarra & Reader 2013). In the case of *A. bipunctata* some combination of thermoregulation and predation, as well as non-random mating (Majerus 1998), covering all three main hypotheses relating to body colour function in animals, appears to influence the frequency of melanic morphs. Other studies have shown that colouration can be subject to opposing selection between thermoregulation and mate choice in *Colias* butterflies (Ellers & Boggs 2003), or in the wood tiger moth between aposematism and either thermoregulation (Lindstedt *et al.* 2009; Hegna *et al.* 2013), immune response (Nokelainen *et al.* 2013) or mate choice (Nokelainen *et al.* 2012).

Whilst there are many possible adaptive functions of body colour, selectively neutral mechanisms may play a role in the maintenance of colour polymorphism (Gray & McKinnon 2007; Protas & Patel 2008). Genetic drift is the random change in allele frequencies at both functional and selectively neutral loci (Freeman & Herron 2007). The influence of genetic drift can be stronger than selection, particularly at small population sizes (Freeman & Herron 2007). When the strength of selection varies temporally or spatially, genetic drift can periodically have more influence on morph frequencies than selection (O'Hara 2005; Oxford 2005). In other cases, the same degree of divergence between populations has been detected at both loci responsible for colour variation and selectively neutral loci, therefore genetic drift could explain the pattern of colour divergence (Hoffman *et al.* 2006; Leinonen *et al.* 2006; Runemark *et al.* 2010; Sánchez-Guillén *et al.* 2011). Gene flow, another selectively neutral process, is the movement and subsequent reproduction of individuals from one population into another (Freeman & Herron 2007). Gene flow between divergent populations can re-introduce colour morphs that have been lost due to selection (or drift) and maintain colour polymorphism (Gray & McKinnon 2007). Selection-migration balance has been reported as important in the maintenance of colour polymorphic populations in the Lake Erie water snake, *Nerodia sipedon* (King & Lawson 1995), in three lizard species that inhabit the White Sands National Monument in New Mexico (Rosenblum 2006), and in the white-phased black bear, *Ursus americanus kermodei* (Hedrick & Ritland 2012).

Rather than the outcome of selection or drift on genetic variation, colour variation could be the result of phenotypic plasticity. The environment can have an important influence on the development of body colour in insects (Whitman & Ananthakrishnan 2009). Variation in the degree of melanism in another ladybird species, *Harmonia axyridis* (the harlequin ladybird) illustrates this point even when colour morphology has a known genetic basis. This species is highly polymorphic for colour pattern, with the background colour being either black with red/orange markings

(melanic morphs) or the opposite (non-melanic morphs) (Wang *et al.* 2009; Michie *et al.* 2010). This colouration is controlled by four main alleles at a single locus (Michie *et al.* 2010). Seasonal changes in the frequencies of melanic and non-melanic morphs have been observed in part of its native range in China and this has been attributed to a non-visual mechanism, with melanic morphs having a selective advantage due to more efficient thermoregulation in winter but being at a disadvantage in summer (Wang *et al.* 2009; Michie *et al.* 2010; Michie *et al.* 2011). Michie *et al.* (2010) and Michie *et al.* (2011) used laboratory experiments to test the effects of rearing temperature on the development of body colour within the genetically determined morph types. These experiments revealed that rearing temperature has a significant effect on the resulting degree of melanism within genotypes. This is best exemplified by the effect of rearing temperature on the non-melanic morph that has red/orange background colouration with black markings. Larvae reared at 14°C always developed 19 markings while at 28°C the average was only 8 markings. This was complemented by a significant decrease in marking size at higher temperatures. The combination of these effects leads to individuals with a reduced area of melanic colouration when reared at higher temperatures and vice versa (Michie *et al.* 2010; Michie *et al.* 2011), and this trend was also observed in field populations (Michie *et al.* 2011). Plastic traits can be adaptive (Via *et al.* 1995) and can create patterns of colour variation that mirror those expected under selection (Alho *et al.* 2010). Therefore, it is important to consider the possible influence of plasticity when interpreting patterns of colour variation.

Biology and colouration of bumble bees

One insect genus that shows large variation in body colour is that of the bumble bees (*Bombus* Latreille). Currently there are approximately 250 recognised *Bombus* species in the world, although more than 2800 formal names have been used to describe species; largely due to the considerable variation and overlap in body colour (Williams 1998). Cameron *et al*. (2007) published a comprehensive phylogeny of the *Bombus* genus using DNA sequence data, which had strong support for the conventional sub-generic system (using morphology, mainly male genitalia). This phylogeny grouped species into 38 subgenera, with subgenera grouped into two sister clades (short-faced and long-faced) that broadly correspond to different facial morphologies and tongue characteristics.

Most bumble bee species have an annual colony life cycle, although bivoltism or perennial nests have been documented in some species (Plowright & Laverty 1984). Fertilized queens emerge in

spring from hibernation to form colonies. These queens will rear broods of workers that take on the duties of foraging and "housekeeping" for the nest. The size of a colony varies between species and is also dependent upon the resources available. Colonies that have been successful in gathering enough resources go on to produce reproductives (queens and/or males), with queens going into hibernation after mating, being the only individuals to survive through the winter and into the following year (Plowright & Laverty 1984; Goulson 2003). Whilst there are greater numbers of species observed in higher altitude and alpine environments, bumble bee species can be found across diverse habitats ranging from the northern temperate zone to lowland tropical forests (Williams 1998).

Bumble bees are large bodied and often brightly coloured flying insects that are covered in a dense layer of hair like extensions of the cuticle, known as pile or pubescence, which functions as insulation (Heinrich 1974; Heinrich 1979). While the underlying cuticle of the insect is black, variation in the colour of the pile leads to different body colour patterns. In a global assessment of colour patterns of 219 non-parasitic bumble bee species; Williams (2007) found that they formed 38 different colour pattern groups. These can be further summarised into three groups (Figure 1.1): A) mostly black (melanic), B) mostly pale yellow-brown or grey and C) the largest group with a pattern of strongly contrasting bands (Williams 2007).

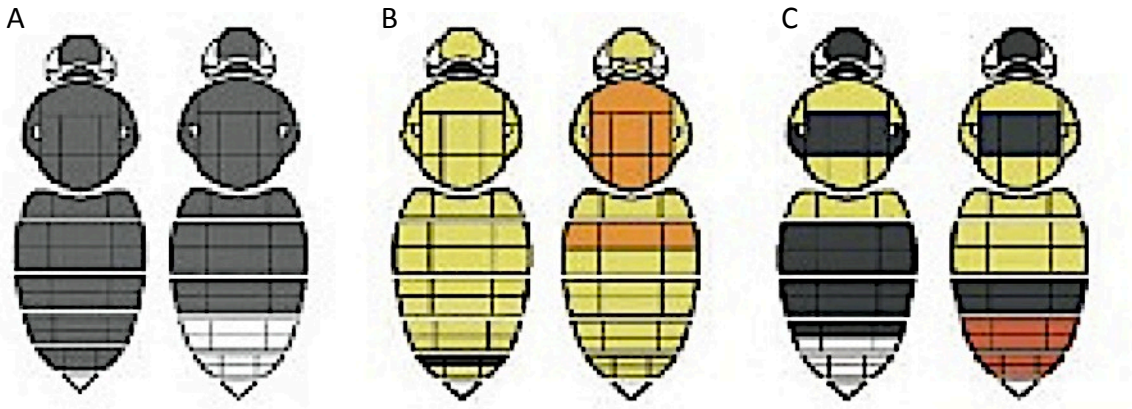


Figure 1.1: the three main bumble bee colour groups: A) mostly black (melanic), B) mostly pale and C) banded. Pictures are from Willams (2007).

Over the global distribution of *Bombus* species, the melanic forms of group A are more common in the Tropics (Williams 2007), counter to the expectations for thermal melanism. This is true even when altitude is taken into consideration and is thought to play a role in thermoregulation or crypsis (Williams 2007), although the advantage of darker colouration for thermoregulation in warmer and more humid environments is unclear. Selection acting on the remaining two groups is

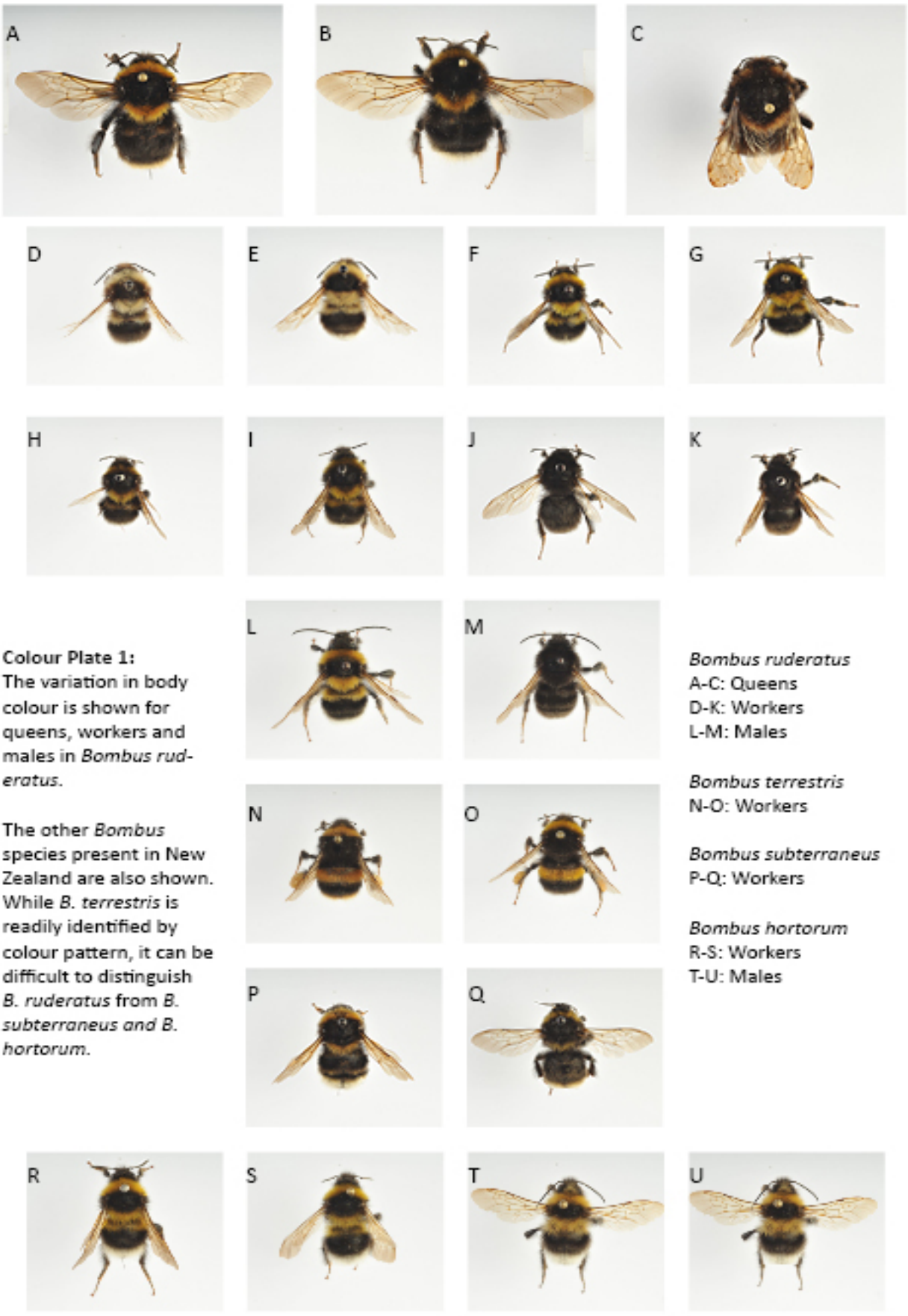
thought to be due to visual mechanisms. The palest colour patterns of group B are mostly found at intermediate northern latitudes and are believed to function as camouflage in temperate grasslands (Williams 2007). In general it is believed that the banded group C, the most common form of colouration in bumble bees, is aposematic (Williams 2007). This is thought to arise because in bumble bees, like other social hymenoptera (e.g. honey bees, wasps and hornets), all females are armed with a venomous sting (Herman & Blum 1981). Müllerian mimicry is therefore thought to be important in the convergence of colour patterns between bumble bee species (Plowright & Owen 1980; Williams 2007; Hines & Williams 2012). A study by Hines & Williams (2012) used DNA sequence data to resolve the phylogenetic relationships between three bumble bee species in South-East Asia. They document complex variation in colour patterns with convergence of even very small areas of colour between species in some areas, suggesting strong selection for mimicry (Hines & Williams 2012). The kleptoparasitic "cuckoo bees" also converge on the same colour patterns as their host species (Goulson 2003).

In addition to the variation in colour patterns among species, colour patterns can be highly variable within species, showing clinal variation over their distribution (Pekkarinen 1979; Plowright & Laverty 1984; Williams 1998). Williams (2007) determined that species had on average three different colour morphologies. Müllerian mimicry can also influence colour variation within a species. Colour polymorphic populations can occur where three bumble bee species are found in sympatry and one species mimics both of the other bumble bee species that have different colour patterns (Plowright & Owen 1980; Hines & Williams 2012). Plowright & Owen (1980) describe the colour polymorphism in *Bombus rufocinctus*, with both red/yellow and black/yellow variants that closely resemble *B. ternarius* and *B. vagans* respectively. Where all three species occur in sympatry, stable polymorphism of both *B. rufocinctus* variants occurs.

Clines of melanism within a species range have been explained as having a thermoregulatory function. Pekkarinen (1979) found a significant correlation between the number of days in spring with a mean temperature between 5°C and 12°C and the percentage of melanic individuals found in *Bombus hortorum* populations in Scandinavia. This suggests that melanism may play a thermoregulatory role for queens foraging at this time. Stiles (1979) advocated that sexual dimorphism in body colour observed in some species has a thermoregulatory basis. Male bumble bees leave the nest and do not return, instead spending the night resting on flowers. As such they do not benefit from the warmth of the nest as female bees do. Therefore, different selective pressures with regards to thermoregulation are expected for male bees compared to females (Stiles 1979). The length of the pile is the most important factor influencing its ability to insulate bees at

night when resting (Stiles 1979); as such male pile length relative to females increased in species found at colder latitudes north of 30°N (Stiles 1979). At these same latitudes male bumble bees also had lighter colouration (increased area of yellow compared to black) relative to females, thought to reduce heating up by reflection of solar radiation to help prevent overheating when in flight during in the day (Stiles 1979).

Whilst the colouration of bumble bee pile is a poor morphological character for species identification, it is thought to be under genetic control. The genetic basis of pile colouration has been described for *Bombus melanopygus*, due to Mendelian inheritance at a single biallelic locus with the allele for red colour dominant over the allele for black (Owen & Plowright 1980; Owen *et al.* 2010). However, there has been no investigation of the actual genes involved in colour development in bumble bees. In addition, the influence of the environment on colour development in bumble bees has not been tested. Temperature has been found to influence development of insect colouration (Goulson 1994; Yamamoto *et al.* 2011; Michie *et al.* 2010). However, this is unlikely to be a factor because bumble bee queens actively incubate their developing brood and the temperature within their insulated colonies is regulated by workers (Heinrich 1979; Goulson 2003). Considerable worker size polymorphism within single colonies is common across bumble bees (Goulson 2003), which appears to be the outcome of differential feeding of larvae by nursing workers (Couvillon & Dornhaus 2009; Couvillon *et al.* 2010). The pigment responsible for yellow colouration in bumble bees has been difficult to characterise, but may be derived from pollen (Hines 2008). Therefore, diet may be one factor that influences colour development in bumble bees.



Colour Plate 1:
The variation in body colour is shown for queens, workers and males in *Bombus ruderatus*.

The other *Bombus* species present in New Zealand are also shown. While *B. terrestris* is readily identified by colour pattern, it can be difficult to distinguish *B. ruderatus* from *B. subterraneus and B. hortorum*.

Bombus ruderatus
A-C: Queens
D-K: Workers
L-M: Males

Bombus terrestris
N-O: Workers

Bombus subterraneus
P-Q: Workers

Bombus hortorum
R-S: Workers
T-U: Males

Research objectives and thesis framework

In New Zealand as throughout its distribution, the ruderal or large garden bumble bee *Bombus ruderatus* shows considerable variation in body colour (Donovan 2007). This variation is due to the different degree and pattern of melanism between individuals and is observed across both queens and workers as well as in males. Individuals range from the "banded" morph that has a yellow on black banding pattern with a white tail, with intermediate forms through to the "black" or "melanic" morph that has black pile covering the entire dorsum. The three other bumble bee species found in New Zealand, *B. terrestris, B. hortorum* and *B. subterraneus* generally have little variation in coat pile colour (Donovan 2007) (COLOUR PLATE 1).

There are no native *Bombus* to Australasia, and the initial introduction of bumble bees into New Zealand was made to facilitate red clover (*Trifolium pratense*) pollination, which was the main fodder crop at the time (Hopkins 1914). A series of deliberate introductions from England to New Zealand in 1885 and 1906, near Christchurch and Lincoln respectively, resulted in the naturalisation of the four European bumble bee species present in New Zealand today (Hopkins 1914; Macfarlane & Gurr 1995). Whether body colour patterns of *B. ruderatus* converge between species at different locations or follow a cline in environmental conditions is unknown in New Zealand. As *B. ruderatus* is an introduced species, initial colour morph frequencies may have been affected by genetic drift in small founding populations before adaptation over a cline was established. However, it is reasonable to expect the establishment of a cline due to selection given the species rapid range expansion in the South Island (Macfarlane & Gurr 1995) following its introduction more than 100 years ago. There are examples where selection has caused the establishment of clinal variation in a relatively short time frame following introduction in *Drosophila subobscura* (Huey *et al.* 2000; Gilchrist *et al.* 2001; Gilchrist *et al.* 2004), rabbits (Williams & Moore 1989) and in flowering plants (Maron *et al.* 2004; Montague *et al.* 2008).

To determine the mechanisms that are contributing to the maintenance of colour polymorphism in *B. ruderatus* in New Zealand, my research was targeted around three main questions:

1. Is body colour in *B. ruderatus* associated with a cline in environmental conditions (non-visual mechanism) and/or the frequency of similarly coloured *Bombus* species (visual mechanism)? If so, body colour may be under selection, with polymorphism being a balance between local adaptation and gene flow.
2. Is body colour in *B. ruderatus* associated with differentiation at selectively neutral loci between populations rather than environmental variables? If so, the current pattern of

body colour polymorphism may be a result of gene flow and genetic drift, rather than selection.

3. Is body colour in *B. ruderatus* influenced by phenotypic plasticity? If so, body colour polymorphism may be the result of environmental influences on development.

In Chapter 2, I used NIWA's climate database to assess the differences in various climatic conditions between bumble bee sampling sites in the South Island. I also assessed the relative abundance of the four introduced *Bombus* species at each of the sampling locations. The climate characteristics of the regions where samples were taken from and the differences in relative abundance of each species collected at those sites are outlined. These data will form the basis needed to compare colour morph frequencies in relation to question 1 in Chapter 4.

In chapter 3, I will use microsatellite marker data to quantify the genetic structure of *B. ruderatus* populations in the South Island. This will allow the relative levels of gene flow and connectivity between populations to be estimated. By quantifying differentiation at selectively neutral loci I will also be able to assess the influence of genetic drift. Levels of relatedness between individuals will also be determined. These data will form the basis needed to address questions 2 and 3 in Chapter 4.

In Chapter 4, I will use digital photography to measure the dorsal body colour of sampled *B. ruderatus.* I will then use this data to test for a correlation with the climate, abundance and genetic data. This approach allows the relative influence of possible selective and neutral factors influencing the variation of body colour to be evaluated in the context of both questions 1 and 2. I will also assess how variation in body colour is associated with relatedness between individuals and look for a seasonal change in the frequency of colour morphs as indicators of phenotypic plasticity to satisfy question 3.

Finally, in Chapter 5, I will provide a synthesis of my findings and suggest future directions for research to improve our understanding of bumble bee colour variation.

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CHAPTER 2: Distribution of *Bombus* species and regional differences in climate

The initial introductions of bumble bees into New Zealand in 1885 and 1906 were made to facilitate red clover (*Trifolium pratense*) pollination, which was the main fodder crop at the time (Hopkins 1914; Macfarlane & Gurr 1995). Currently, bumble bees are used as pollinators of several flowering crops grown in New Zealand, including kiwifruit (*Actinidia deliciosa*), both white (*T. rapens*) and red clover, lucerne (*Medicago sativa*) and glasshouse crops such as tomatoes (Howlett & Donovan 2010). As bumble bees are manageable through the use of man-made nest boxes (Donovan & Weir 1978) they provide one of the most practical alternatives to honey bees as commercial pollinators in New Zealand (Howlett & Donovan 2010). Whilst introduced bees have the potential to negatively interact with native bee species (Goulson 2003), there appears to be very little competition for either floral resources or nesting sites between bumble bees and native bees in New Zealand (Donovan 1980). However, bumble bees may facilitate the spread of invasive exotic weeds (Goulson 2003) such as tree lupins (*Lupinus arboreus*) (Goulson & Rotheray 2012).

Bombus terrestris has become wide spread in both the North and South Islands (Macfarlane & Gurr 1995). It is the only species in New Zealand in the short-tongued group and as such has a more generalized foraging preference than the other three long-tongued species (Goulson & Hanley 2004; Goulson *et al.* 2008a). Goulson & Hanley (2004) found that *Bombus terrestris* was the only *Bombus* species that was present across the entire range of habitat types they surveyed, including native vegetation where the other species are absent. However, the abundance of *B. terrestris* is linked to habitat type, with the lowest abundances recorded in native vegetation and the highest abundances recorded along lake margins, rivers and rough pasture (Goulson & Hanley 2004).

Macfarlane & Gurr (1995) recorded *B. subterraneus* as confined to inland areas of Otago and Canterbury in the South Island. This range appears to have further decreased to a small region within 2 km of the lake margins at Lake Tekapo, Twizel and Wanaka (Goulson & Hanley 2004). As such, the presence of *B. subterraneus* is strongly linked to lake margin habitat and it was also only found at sites where all of the other *Bombus* species were present (Goulson *et al.* 2006).

Bombus hortorum has a relatively wide distribution and is found in both the North and South Islands, although appears restricted to cooler and moister districts (Macfarlane & Gurr 1995). Goulson & Hanley (2004) found that *B. hortorum* was most abundant in improved/semi-improved pasture. They also found that *B. hortorum* was present in all of the habitat types where *B. ruderatus* was found, although *B. ruderatus* showed a preference for lake margins and rough pasture (Goulson & Hanley 2004). *Bombus ruderatus* also has a relatively wide distribution and is found in both the

North and South Islands, being most abundant in lightly settled districts with the warmest and driest climates in both islands (Macfarlane & Gurr 1995). Macfarlane & Gurr (1995) listed *B. ruderatus* as more common than *B. hortorum*; however the distribution of *B. ruderatus* in the central South Island appears to have reduced since 1995 (Goulson & Hanley 2004). Interestingly, the pattern in relative abundance of these species now seems to mirror that observed in the UK, with *B. terrestris* most common, followed by *B. hortorum* and *B. ruderatus,* then *B. subterraneus* as the rarest (extinct in the UK) (Goulson *et al.* 2006).

In New Zealand, the three long-tongued species were found to be heavily reliant upon two introduced plant species (*Trifolium pratense* (red clover) and *Echium vulgare* (vipers bugloss)) for both nectar and pollen (Goulson & Hanley 2004; Lye *et al.* 2010). The decline of *B. ruderatus* and other long-tongued species in the UK is thought to be linked to the reduction and fragmentation of habitat, in particular a reduction in the distribution and abundance of Fabaceae (*i.e.* red clover) (Goulson *et al.* 2005; Carvell *et al.* 2006). A change in the composition of *Bombus* species utilizing red clover fields, with a large increase in the relative abundance of short-tongued species at the expense of long-tongued species has been documented in Denmark (Dupont *et al.* 2011) and Sweden (Bommarco *et al.* 2012). Agricultural practises have changed in New Zealand since 1960, with a reduction in grassland areas and rough pasture and an increase in improved pasture (MacLeod & Moller 2006). There has also been a shift away from the traditional use of clover to fix nitrogen in soils and an increase in the use of maize and cereal silage to supplement feed (MacLeod & Moller 2006), both of which suggest a decline of red clover use in New Zealand.

With changes to the agricultural landscape in New Zealand, gardens could provide important habitat for bumble bees. Garden habitat can be beneficial for biodiversity conservation (Goddard *et al.* 2010), and can be important to bumble bees by providing floral resources (Goulson *et al.* 2002) and nesting sites (Osborne *et al.* 2008; Goulson *et al.* 2010; Lye *et al.* 2012). However, gardens are mainly utilised by short-tongued species (Goulson *et al.* 2006) and urbanisation has been found to negatively affect native bee species diversity in general (Martins *et al.* 2013) as well as bumble bee species diversity (Ahrné *et al.* 2009) and nest density (Jha & Kremen 2013). In the two garden habitats surveyed by Goulson & Hanley (2004) the only species present was *B. terrestris.* The importance of garden habitat for *Bombus* species in New Zealand is currently poorly understood.

Whilst previous survey work by Goulson & Hanley (2004) outlines the distributions of *Bombus* species in the central South Island (concentrated at sites within the previously outlined distribution of *B. subterraneus*), how their distributions may have changed since is unclear. Little was known

about their distribution across the upper South Island and this distribution may have changed since the survey by Macfarlane & Gurr (1995). The principle aim for this part of the study was to locate and sample individuals from several populations of *B. ruderatus* in the South Island. In order to represent a range of climatic conditions that potentially influence colour polymorphism in *B. ruderatus*, I searched for populations across the South Island from the southern most site at Queenstown to the northern most at Nelson. Regional differences in temperature, sunshine, rainfall and humidity as measures of climate were determined. These measures relate to specific hypotheses about the selective maintenance of colour polymorphism due to either thermoregulation or desiccation resistance (see Chapter 1). As Müllerian mimicry is thought to be important in the evolution of bumble bee colouration (Plowright & Owen 1980; Williams 2007; Hines & Williams 2012), the relative abundance of each *Bombus* species was also recorded so that any potential influence on body colour in *B. ruderatus* could be assessed. The differences between sites are discussed and how these are linked to body colour will be evaluated in Chapter 4.

Methods

Field collection

Sites were sampled in 2012 and 2013; all sites being sampled in January with the exception of Nelson-Motueka and Lake Coleridge (both 2012) that were sampled in early February. In 2012, two sites were re-sampled in March (Christchurch- Mona Vale garden & Lake Tekapo). All of the sampling was conducted between 08:00 and 17:00 and only in weather favourable for bumble bee activity (Frankie *et al.* 2002). Sites were selected based upon the presence of suitable forage for bumble bees and each site comprised of an area of wild flowering plants situated on either a lake or a roadside margin. As Goulson & Hanley (2004) only searched two garden habitats, I also conducted two searches in garden habitats (Mona Vale in Christchurch and Queenstown botanical gardens) to further assess the relative abundance of bumble bee species in urban environments in New Zealand. An hour count was performed at each site, in which all individuals were caught and kept in plastic containers until the end of the hour period when each bee was tentatively identified by close morphological inspection. The relative abundance of each species was recorded and then all of the individuals that were not *B. ruderatus* were released. Only *B. ruderatus* workers were taken so any queens or males of this species were also released. Collection of *B. ruderatus* workers continued at the site until 30 to 35 individuals were obtained. These individuals were euthanized by freezing in order to minimise clumping of the pile that I found to occur with moisture build up in killing jars. Leg tissue was taken from each individual and stored in 99% ethanol for DNA extraction. All bees

collected had their species identification confirmed using a PCR method (See Methods Chapter 3: *Species identification*).

Climate data

Data for regional differences in climate were obtained from NIWA's National Climate Database (CliFlo). For each variable, data were used from the weather recording station that was closest to the site where the samples were collected (Table 2.1). The average daily minimum and maximum air temperatures for each month between the years 1993 to 2012 were compared for Alexandra, Wanaka, Queenstown, Nelson, Oamaru, Lake Tekapo, Twizel and Christchurch. The average total rainfall and relative humidity for each month between the years 1993 to 2012 were compared for the same regions as above and for total rainfall Lake Coleridge was also included (this was the only measurement recorded at the Lake Coleridge weather stations). The total sunshine hours for each month between the years 1971 and 2000 were compared for Alexandra, Nelson, Oamaru, Lake Tekapo, Queenstown and Christchurch (data were unavailable for Twizel). Two other measures, "wet days" and "cool period" are not included here as they were correlated with some of the other climate measures causing co-linearity in the models in Chapter 4 (Appendix 1).

Table 2.1: Weather station information

Name	Agent Number	Network Number	Latitude (dec.deg)	Longitude (dec.deg)	Height (m)
Alexandra	5578	I59236	-45.246	169.38956	150
Christchurch Aero	4843	H32451	-43.493	172.537	37
Lake Tekapo, Air Safaris	4970	H40041	-44.002	170.441	762
Nelson Aero	4241	G13222	-41.299	173.226	2
Oamaru Airport	5141	I41901	-44.976	171.083	30
Oamaru Airport Aws	5142	I41902	-44.973	171.081	30
Queenstown	5446	I58061	-45.037	168.663	329
Queenstown Aero	5450	I58074	-45.024	168.735	349
Twizel 2	4997	H40213	-44.264	170.103	457
Twizel Substation	4996	H40212	-44.277	170.098	451
Wanaka	5223	I49711	-44.705	169.146	314
Wanaka Aero Aws	7426	I49723	-44.724	169.244	352
Wanaka, Park Hq	5217	I49613	-44.696	169.142	305
L Coleridge Homestead	4669	H31351	-43.348	171.587	514
Lake Coleridge	4670	H31352	-43.365	171.529	364

Each measure of climate showed variation between months, but with the same trend across sites. For this reason the values for each measure were averaged over the months of October through to March, which shows the difference in each variable between sites over the period when queens, workers and males are flying. R (R Development Core Team 2008) was used to perform a two factor

ANOVA with sites as a factor and years as a random factor for each measure of climate, followed by pairwise comparisons using the post-hoc LSD method.

Results

Relative abundances

The relative abundance of each *Bombus* species varied across sites in the South Island (Figure 2.1). There was a significant difference among sites between the relative abundance of *B. terrestris, B. hortorum* and *B. ruderatus* (χ^2= 792, P<0.001, D.F.= 30). The most abundant species was *B. terrestris* (786 bees recorded), which was found at all the sites. At the Waipara, Lake Tekapo, Alexandra and both Queenstown sites, *B. terrestris* was clearly the most abundant species present (Table 2.2). The second most abundant species was *B. hortorum* (467 bees), which had greater or similar abundances to *B. terrestris* at the Nelson, Lake Coleridge, Waitaki River and Wanaka sites. At the Twizel, Lake Tekapo and Alexandra sites, *B. hortorum* was either low in abundance or absent (Table 2.2). *Bombus ruderatus* (224 bees) was the third most abundant species, although at two sites (Brightwater and Twizel) it was the most abundant species. At the Motueka, Christchurch-Mona Vale garden, Waipara and Queenstown-botanic garden sites *B. ruderatus* was either very low in abundance or absent (Table 2.2). The rarest species was *B. subterraneus* (9 bees). The range of *B. subterraneus* was restricted and it was only found in low abundance at Lake Tekapo and Wanaka.

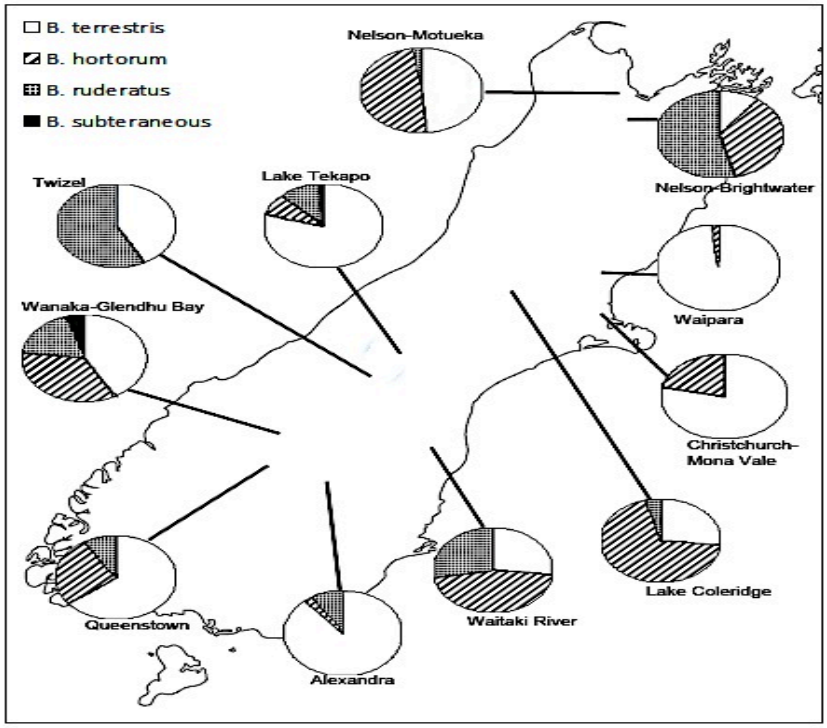

Figure 2.1: Distribution and relative abundance of the four *Bombus* species in the South Island, New Zealand.

The relative abundance of each species is similar between the sampling in January and March at Lake Tekapo. There was a shift in the relative abundance of each species recorded at the Christchurch-Mona Vale site between January and March. In January *B. terrestris* was the most abundant species (55 bees) and *B. hortorum* the second most abundant (15 bees), the other species were not present. In March *B. hortorum* was the most abundant (40 bees), *B. terrestris* was the second most abundant (15 bees) and a single *B. ruderatus* worker was recorded (Table 2.2).

Table 2.2: Abundance of each Bombus species from the hour count performed at each location. The most common forage species at each site is noted as either *Echium vulgare* or *Trifolium pratense*. Gardens were not dominated by a single forage species, containing a wide variety of introduced flowering plants. All locations were sampled in January or early February, unless stated otherwise.

Location	*terrestris*	*hortorum*	*ruderatus*	*subterraneus*	Forage spp.
Queenstown Gardens 2012	34	25	0	0	-
Queenstown 2012	58	24	8	0	*E. vulgare*
Wanaka 2012	30	49	3	0	*E. vulgare*
Glendhu Bay 2013	60	50	26	7	*E. vulgare*
Alexandra 2013	127	1	13	0	*E. vulgare*
Waitaki River 2013	30	51	31	0	*E. vulgare*
Twizel 2013	38	0	52	0	*E. vulgare*
Lake Tekapo 2012	70	7	10	1	*E. vulgare*
Mona vale 2012	55	15	0	0	-
Lake Coleridge 2012	39	49	6	0	Both
Lake Coleridge 2013	38	98	6	0	Both
Waipara 2013	75	1	0	0	*E. vulgare*
Nelson-Brightwater 2013	10	35	53	0	*T. pratense*
Nelson-Motueka 2012	21	21	1	0	*T. pratense*
Lake Tekapo March 2012	86	1	14	1	*E. vulgare*
Mona Vale March 2012	15	40	1	0	-
Totals	**786**	**467**	**224**	**9**	

Regional differences in Climate

The average daily maximum temperature differed significantly between regions ($F_{7, 130} = 52.96$, $P < 0.0001$). Alexandra had the highest average daily maximum at 22.6 °C, which was significantly different from all other regions. Twizel, Wanaka, Queenstown and Nelson did not differ significantly from one another, with an average between 21.1 °C and 20.5 °C, while Christchurch with an average of 20.1°C differed significantly from Twizel. There was no significant difference between the two coldest regions, Lake Tekapo at 18.8 °C and Oamaru at 18.1 °C, which differed significantly from the other regions (Figure 2.2A).

The average daily minimum temperature differed significantly between regions ($F_{7, 129} = 106.25$, $P < 0.0001$). Nelson had the highest average daily minimum at 11.4 °C, which differed significantly from all other regions. Christchurch had the second highest average daily minimum at 9.3°C, which differed significantly from all other regions. Queenstown, Oamaru, Wanaka and Alexandra did not differ significantly from one another, with an average daily minimum between 8.6 °C and 8.0 °C. The second coldest region was Twizel with an average of 6.6°C which differed significantly from all other regions. The coldest region was Lake Tekapo with an average of 5.9°C which differed significantly from all other regions (Figure 2.2B).

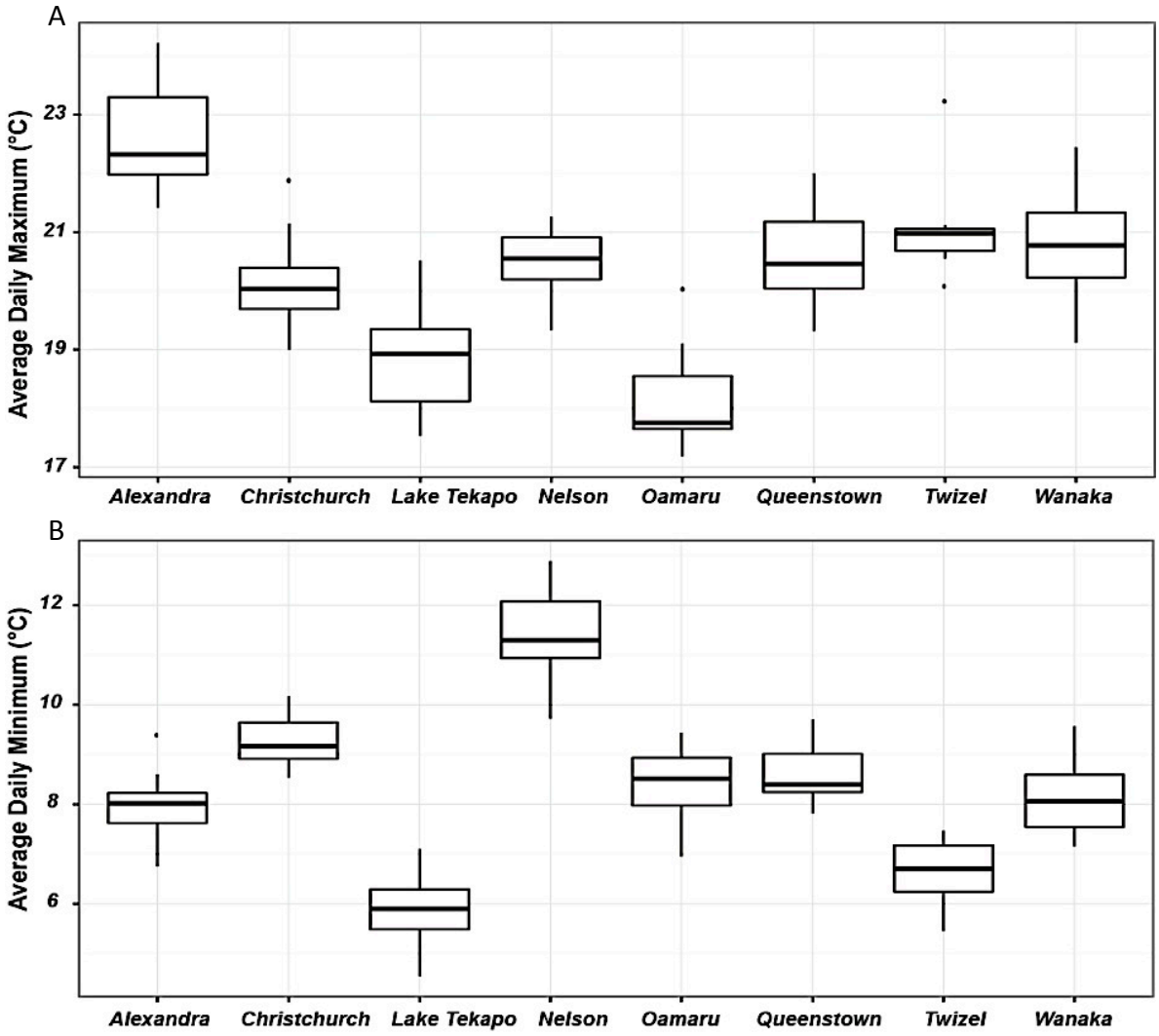

Figure 2.2: Differences in measures of temperature (°C) between regions for the months October through to March. A) Average daily maximum and B) average daily minimum across regions. Data were averaged for the period from 1993 to 2012. The heavy bar is the mean, with the box representing the 25th and 75th percentiles. The lines extend to values within 1.5 * the inter-quartile range, points are values outside this range and may be outliers.

The average total sunshine hours differed between regions ($F_{6, 26} = 7$, $P < 0.0001$). Nelson and Lake Tekapo had the highest average total sunshine hours with means of 235.5 and 230.5 hours respectively. Alexandra, Christchurch, Queenstown and Wanaka had similar averages ranging between 212 and 208 hours. Oamaru had the lowest average total sunshine hours with a mean of 171.8 hours (Figure 2.3).

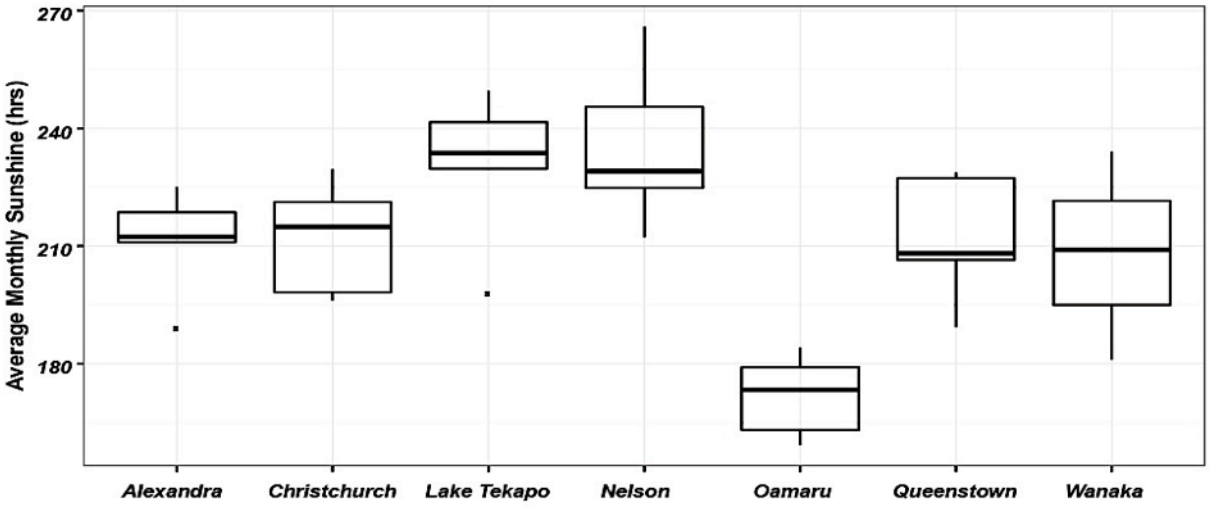

Figure 2.3: Differences in the average monthly sunshine hours between sites for the months October through to March. Data were averaged for the period from 1971 to 2000. No data for Twizel was available. The heavy bar is the mean, with the box representing the 25th and 75th percentiles. The lines extend to values within 1.5 * the inter-quartile range, points are values outside this range and may be outliers.

The average total rainfall differed significantly between regions ($F_{8, 145} = 16.48$, $P < 0.0001$). Alexandra had the lowest average total rainfall at 33.4 mm, followed by Oamaru at 42.2 mm, Christchurch at 43.1 mm and Lake Tekapo at 43.3 mm. Wanaka had an average of 47.7 mm, which was significantly higher than Alexandra. The regions with highest average rainfall were significantly different from the others with Twizel at 58.8 mm, Lake Coleridge at 63 mm, Nelson at 72.6 mm and Queenstown with the highest at 74.1 mm (Figure 2.4A).

The average monthly relative humidity differed significantly between regions ($F_{7, 134} = 28.5$, $P < 0.0001$). Oamaru had the highest average relative humidity at 78.9 %, which differed significantly from all other regions. Christchurch, Nelson, Queenstown, Alexandra and Lake Tekapo had averages ranging from 75.8 to 70.1 %. The lowest average relative humidity was recorded at Twizel and Wanaka as 67.5 and 67.2 % respectively (Figure 2.4B).

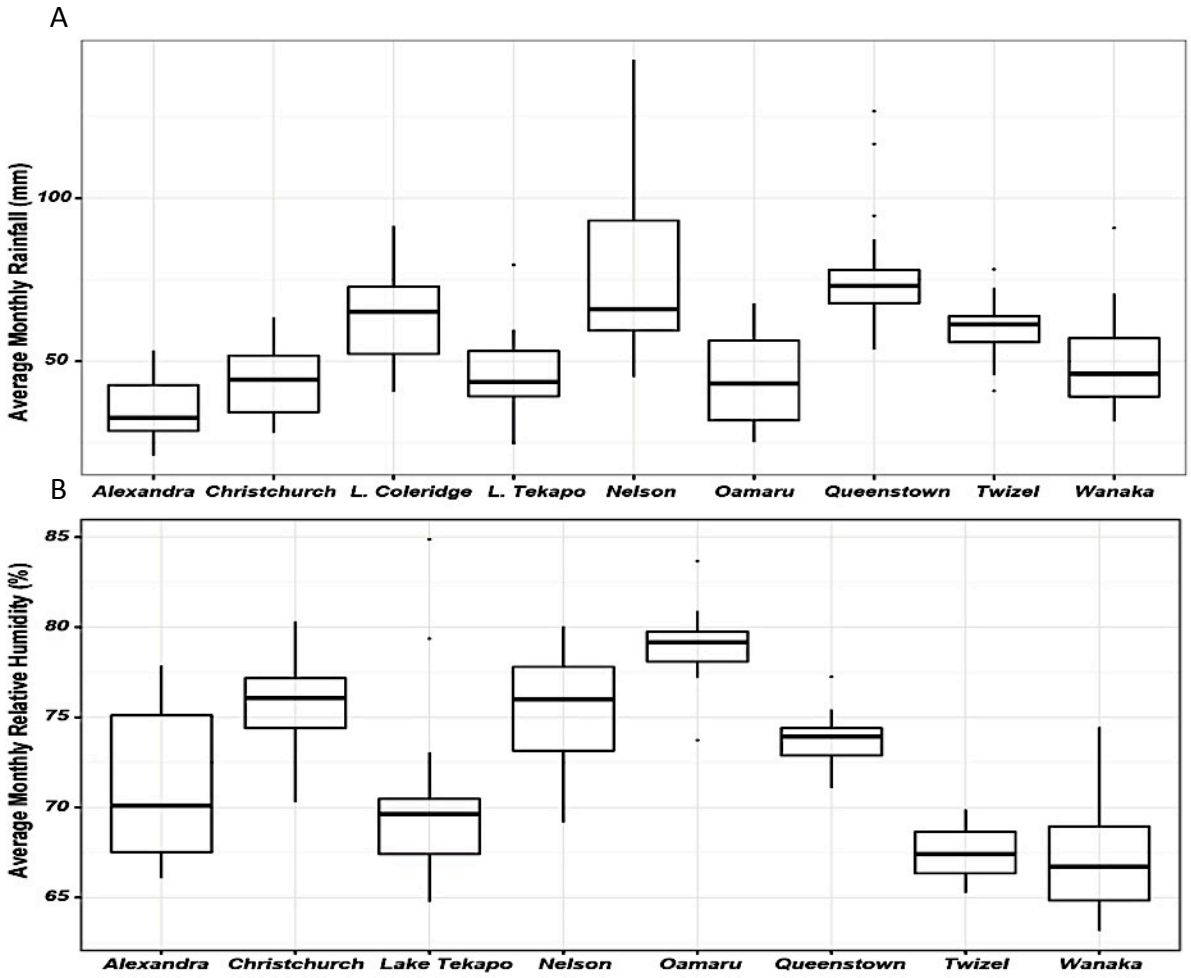

Figure 2.4: Differences in measures of precipitation and humidity between regions for the months October through to March. A) Average monthly rainfall (mm) and B) average monthly relative humidity (%) across regions. Data were averaged for the period from 1993 to 2012. The heavy bar is the mean, with the box representing the 25th and 75th percentiles. The lines extend to values within 1.5 * the inter-quartile range, points are values outside this range and may be outliers.

Discussion

The overall trend in abundance and distribution of *Bombus* species in the South Island was similar to the findings of Goulson & Hanley (2004), with the most abundant species being *B. terrestris* followed by *B. hortorum,* then *B. ruderatus* and finally *B. subterraneus* as the rarest. As was expected, *B. subterraneus* was only found in low abundance near lake margin habitat and only at sites where all other *Bombus* species were present. *Bombus terrestris* was found at all sites and was in many cases the most abundant species at a site. The sites where *B. terrestris* was recorded with a relatively low abundance could be due to differences in forage availability, with sites where *Trifolium pratense* was very common being suited to the more specialised long-tongued species (Table 2.2).

There were significant differences for each of the measures of climate between regions. Temperatures varied with a difference of 4.5 °C and 5.5 °C between the highest and lowest average daily maximum and minimum temperatures respectively. The average number of sunshine hours also varied from 235.5 to 171.8, with Oamaru having fewer sunshine hours than the other regions. Sites varied in the average relative humidity from 79 to 67 %. There was large variation in the average monthly total rainfall between regions with a difference of 40 mm between Alexandra and Queenstown.

Macfarlane & Gurr (1995) concluded that *B. hortorum* is restricted to cooler, wetter districts and *B. ruderatus* is more common in the drier, warmer inland regions of the South Island. Consistent with this, the sites with the lowest abundance of *B. hortorum* where *B. ruderatus* was also present were Alexandra, Lake Tekapo and Twizel (Table 2.2). These sites are characterised by having a comparatively dry climate, with Alexandra and Lake Tekapo having on average relatively low monthly rainfall (Figure 2.3) as well as all three sites having a comparatively low average relative humidity (Figure 2.3) and on average fewer wet days per month than the other sites (Appendix 1). In comparison, the sites where *B. hortorum* had a greater relative abundance were Queenstown, Wanaka, Waitaki River and Lake Coleridge. Most of these sites have a climate with relatively high rainfall, humidity (Figure 2.3) and average number of wet days (Appendix 1). Of the two Nelson sites sampled, the Motueka site had a relatively high abundance of *B. hortorum* and a very low abundance of *B. ruderatus,* whereas at the Brightwater site *B. ruderatus* was the most abundant species sampled (Table 2.2). Both of these sites were characterised by a large area of *Trifolium pratense* as the most abundant forage species (Table 2.2). Climatic differences at this scale could not be investigated using the CliFlo database, however as Brightwater is an inland site and the Motueka site is situated comparatively close to the coast this difference could be due to a similar climatic trend over a smaller spatial scale.

Urbanization has been found to negatively affect bumble bee species diversity (Ahrné *et al.* 2009). Of the two sites that can be classified as garden environments, both showed either very low abundance or the absence of *B. ruderatus* and *B. subterraneus,* while *B. hortorum* and *B. terrestris* were present (Table 2.2). Similarly, the site in Wanaka situated at the edge of the town also had a low abundance of *B. ruderatus* and *B. subterraneus* was absent*,* compared to their abundances recorded further around the lake (and away from the town) at Glendhu Bay (Table 2.2). Goulson & Hanley (2004) sampled at two garden sites, where they found only *B. terrestris* present. A possible explanation for the apparent difference in species composition between rural and urban environments could be forage specialisation of some species. Fabaceae species are known to be

important food sources for long-tongued bumble bees (Goulson *et al.* 2005; Goulson *et al.* 2008a). There is a strong specialisation on *T. pratense* and *Echium vulgare* by the long-tongued species in New Zealand (Goulson & Hanley 2004; Lye *et al.* 2010). A lower abundance of these key forage plant species in gardens could explain why *B. ruderatus and B.* subterraneus are not found in abundance in these habitats. The relatively high abundance of *B. hortorum* in the Queenstown botanical gardens and the Mona Vale gardens site in March suggests that specialisation by long-tongued species isn't the only factor. These gardens included an abundance of a *Mentha* species, *Linaria purpurea* and *Digitalis purpurea* on which most of the *B. hortorum* were recorded foraging.

The distributions and abundances of both *B. ruderatus* and *B. subterraneus* have declined in the South Island since the survey by Macfarlane & Gurr (1995). The decline in some native bumble bee species has been well documented in Europe and North America (Goulson *et al.* 2008b; Williams & Osborne 2009; Cameron *et al.* 2011; Brown 2011). The major factors that have been attributed to these declines include the loss of habitat associated with both floral resources and nesting sites, shifting agricultural practises, introduction of diseases, and genetic factors associated with habitat fragmentation (Goulson *et al.* 2005; Goulson *et al.* 2008; Williams & Osborne 2009, Cameron *et al.* 2011). Williams *et al.* (2009) found three major trends in characteristics of bumble bee species in decline; they had a narrower climatic range, susceptibility is greater when species are closer to their climatic range edge, and they had later emergence times. Of the four species in New Zealand, the less common species *B. ruderatus* and *B. subterraneus* have emergence times later than those of *B. terrestris* and *B. hortorum* (Donovan & Wier 1978). The use of red clover has declined in New Zealand agriculture since 1960 (MacLeod & Moller 2006). Species emerging later in the season may be particularly affected by the shift in agricultural practise from hay making (linked to the later flowering of *Trifolium pratense*) to the use of silage (Fitzpatrick *et al.* 2007).

In Summary, these results show that climatic conditions and the relative abundance of *Bombus* species vary between sampling locations in the South Island. This provides the basis for testing hypotheses relating to the selective maintenance of body colour polymorphism in *B. ruderatus*. Like the recent study of *Bombus* in the central South Island by Goulson & Hanley (2004), I found that the abundances and distributions of *B. ruderatus* and *B. subterraneus* have declined since the survey by Macfarlane and Gurr (1995). This may be linked to the change in New Zealand's agricultural landscape, with a reduction in the use of *T. pratense*. Gardens appear to provide floral resources for foraging bumble bees including species with long corollas that were utilised by *B. hortorum.* Although *B. ruderatus* seems to be negatively impacted by urbanisation, it remains locally abundant at some rural locations. As selectively neutral processes may also affect variation in body colour, I

will next examine variation in microsatellite markers in Chapter 3, so that both selective and neutral influences can be evaluated in Chapter 4.

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CHAPTER 3: Genetic structure of *Bombus ruderatus* populations in the South Island, New Zealand.

One mechanism that can maintain variation between populations across a species range is divergent selection when selection pressures are spatially heterogeneous (Hedrick 1986). This occurs when populations experience different local conditions, consequently leading to different selection pressures and divergence through adaptation to these local conditions. The pattern of local adaptation resulting from spatially heterogeneous selection is dependent on the balance between selection and gene flow (Lenormand 2002). Models predict that the degree of heterogeneity in the landscape in combination with the strength of gene flow between locally adapted populations will determine resulting levels of polymorphism (Star *et al.* 2007; Star *et al.* 2008). There are many examples of selection acting upon body colour or correlated traits (for review see True 2003; Roulin 2004; Gray & McKinnon 2007). Thus, patterns of colour polymorphism observed within populations may be the result of a balance between divergent selection and migration between populations (Gray & McKinnon 2007). Alternatively, divergence between populations may result from genetic drift, which is the random change in allele frequencies across both selectively neutral and functional loci (Freeman & Herron 2007), and polymorphism may be the result of a balance between genetic drift and migration between populations. This means that estimating patterns of gene flow between populations that are affected by divergent selection and/or genetic drift is crucial in understanding how variation is maintained within those populations.

The level and pattern of genetic differentiation among populations (*i.e.* genetic structure) at selectively neutral loci can be assessed using microsatellite markers (Balloux & Lugon-Moulin 2002). This structuring can be used to determine the relative levels of gene flow between populations (Lowe & Allendorf 2010). Microsatellite markers are ideal for assessing the relatively recent evolutionary processes that influence genetic differentiation between populations, due to their fast mutation rate, codominance and high degree of polymorphism (Sunnucks 2000; Selkoe & Toonen 2006). They are also particularly good at assessing levels of kinship (Queller *et al*. 1993), which can be informative for studies of social hymenoptera. Whilst development of microsatellite markers can be expensive and time consuming, loci for *Bombus* species have already been developed (Funk *et al.* 2006), making microsatellites the ideal markers for this study.

Several studies have assessed the genetic structure of *Bombus* populations, often in the context of conservation of species that have undergone declines in their native ranges. The genetic structure of *B. ruderatus*, however, has not been investigated in its native range in Europe, despite being in

decline in the UK. Bumble bee species in decline tend to have lower levels of genetic diversity and higher levels of differentiation between populations than species that are stable (Darvill *et al.* 2006; Ellis *et al.* 2006; Charman *et al.* 2010; Cameron *et al*. 2011; Lozier *et al*. 2011). Patterns vary between different species, although most non-declining species exhibit non-significant levels of genetic differentiation between populations in their native range over land in Europe (e.g. Estoup *et al.* 1996 -*B. terrestris*, F_{ST}=0.005; Ellis *et al.* 2006 -*B. pascuorum*, F_{ST}=0.001) and in the USA (Lozier *et al*. 2011 -*B. vosnesenskii,* G_{ST}=0.006 and *B. impatiens*, G_{ST}=0.003). However, in some cases barriers to gene flow have been discovered. Goulson *et al.* (2011) found that bodies of open water between island populations of *B. hortorum* in Scotland acted as significant barriers to gene flow (F_{ST}=0.16, CI=0.13-0.2). Similarly, island populations of *B. terrestris* in Europe were significantly differentiated from those on the mainland (Estoup *et al.* 1996). In another study, the genetic structure of *B. vosnesenskii* in California revealed that urbanization measured as the area of impervious cover created barriers to regional gene flow (Jha & Kremen 2013).

Following introduction to New Zealand in at least one of two release events in Canterbury, *B. ruderatus* is thought to have spread throughout the South Island rapidly (Macfarlane & Gurr 1995). The genetic population structure of *B. ruderatus* in New Zealand will therefore reflect the history of colonisation and initial range expansion from the introduction area in Canterbury, combined with subsequent gene flow, selection and genetic drift. Although the genetic structure of populations is usually determined using selectively neutral loci, selection can influence the genetic structure of populations at neutral loci through influencing the effective migration rate, as selection against maladapted migrants will result in decreased gene flow between populations (Charlesworth *et al*. 2003). Levels of differentiation between *B. ruderatus* populations in New Zealand are expected to be relatively low, even if they have been isolated from one another since the introduction, given their shared ancestry. Lye *et al.* (2011) compared microsatellite variation between *Bombus* species sampled in their source populations in the UK and Twizel in New Zealand. They estimated that the effective number of *B. ruderatus* individuals introduced to New Zealand was between 6 and 67, with the most likely number being 14 (Lye *et al.* 2011). The rapid expansion of populations may result in increased differentiation between populations. As population sizes are smaller at the edge of an expanding species range, genetic drift can become a strong evolutionary force at the expansion's wave front (Excoffier & Ray 2008). This can lead to a change in allele frequencies between the initial population and new populations, as alleles at the wave front change in frequency by chance, a phenomenon known as 'surfing' (Excoffier & Ray 2008).

The genetic structure of *B. ruderatus* in New Zealand has not yet been investigated. However, Schmid-Hempel *et al.* (2007) sampled several populations of *B. terrestris* throughout the South Island to determine the origin of the introduction of *B. terrestris* to Tasmania. They found no significant pairwise F_{ST} values between populations within the South Island and an overall non-significant F_{ST} of 0.06, revealing no significant genetic differentiation from Kaikoura and the West Coast through to Otago. *Bombus terrestris* is distributed throughout the South Island, which should facilitate gene flow between populations. In comparison, *B. ruderatus* can be locally abundant but has a more patchy distribution (Chapter 2); therefore gene flow may be more restricted between *B. ruderatus* populations than between *B. terrestris* populations.

The aim of this chapter is to assess the genetic structure of the *B. ruderatus* populations in the South Island sampled in Chapter 2, using microsatellite markers to determine levels of genetic differentiation between populations. This will allow the relative connectivity and levels of gene flow between populations to be determined. These patterns of differentiation and gene flow are then discussed in the context of the history of colonisation of *B. ruderatus* in New Zealand from the UK. The implications of these patterns for the persistence of *B. ruderatus* populations in the South Island are also considered. Levels of differentiation at selectively neutral loci will provide a benchmark for the levels of divergence between these populations due to random genetic drift. The implications of this in relation to the maintenance of body colour polymorphism within *B. ruderatus* populations are analysed and discussed in Chapter 4.

Methods

Sample collection

Samples were collected from Queenstown in January and Lake Tekapo in January and March in 2012 and samples from Nelson (Brightwater), Wanaka (Glendhu Bay), Alexandra, Waitaki River and Twizel were collected in January 2013. At each site *B. ruderatus* workers were collected as they were foraging and a tentative identification was made by close visual inspection. Collection of *B. ruderatus* continued until 30 to 35 individuals were collected. Leg tissue was taken from each individual and stored in 99% ethanol for DNA extraction. As *B. ruderatus* is cryptic in colouration with *B. hortorum* and *B. subterraneus*, species identification was confirmed using a PCR based method (see Methods: *Species identification*). For more details about sampling locations and method refer to Chapter 2.

DNA extraction

DNA extraction was performed using Chelex®100 resin. The tarsus and tibia from the right side middle leg of each individual was cut into small pieces and placed in a 1.5ml eppendorf tube. To this, 200µl of 5% Chelex®100 resin (Bio-Rad) in PCR water was added and the sample was ground in the solution using a micro pestle. After adding 5µl of 20mg/ml Proteinase K, the sample was incubated at 56 °C for at least 2 hours. The sample was vortexed briefly and then heated at 100 °C for 8 minutes. Following this the sample was vortexed briefly and then centrifuged at maximum speed for 1 minute. The supernatant was then transferred to a new 1.5ml eppendorf tube and the remaining Chelex®100-resin and insect cuticle was discarded.

Species identification

While *B. terrestris* can be readily identified from the other *Bombus* species in New Zealand using colour pattern, lighter coloured morphs of *B. ruderatus* are cryptic in colouration with *B. hortorum* and dark/intermediate morphs can be cryptic with *B. subterraneus.* Therefore each sample was identified to species using the PCR based method described in Stewart *et al.* (2010). This method uses the amplification of part (426 bp) of the mitochondrial *Cytb* locus across all species, using primers CYTBF2 and CYTBR2, combined with a species-specific internal fragment, using primer BHR1 for *B. hortorum* or BSF1 for *B. subterraneus* (Stewart *et al.* 2010). Amplification took place in a 15µl PCR reaction containing 2.5µl DNA extract and 12.5µl master mix that consisted of 1.5µl 10X NH_4 Buffer, 0.85µl 50mM $MgCl_2$, 1.5µl 2mM dNTPs, 0.5µl of each 10mM of CYTB2 primers, 0.18µl of 10mM BHR1 or BSF1 primer, 0.03µl of 5 U/µl BioTaq™ (Bioline) and 7.4µl ddH_2O. The PCR protocol involved an initial denaturing step at 94°C for 4 minutes, followed by 35 cycles of denaturing at 94°C for 30 sec, annealing at 48°C for 30 sec and extension at 72°C for 1 minute, then a final extension of 72°C for 10 minutes. The PCR product was then run at 80V for 45 minutes on a 1.4% agarose gel.

A positive identification was achieved with the presence of a band at approximately 200-240bp indicating species-specific primer amplification (Figure 3.1). When amplification of the non-specific primers occurred (band at ~426bp) and there was no amplification of the species-specific primer the test was scored as negative for that species. As each test either confirms or rules out species identification for that primer, species were identified by a process of elimination, with a negative result for the BHR1 primer (*B. hortorum*) and the BSF1 primer (*B. subterraneus*) a positive identification for *B. ruderatus.* No test was conducted for *B. terrestris* because this species is easily identified using colour pattern morphology.

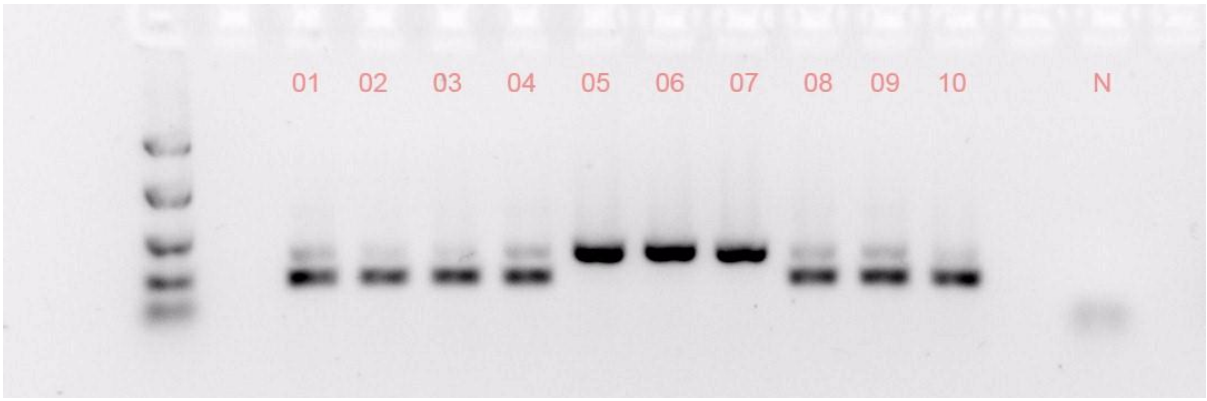

Figure 3.1: Agarose gel electrophoresis of PCR using primers CYTBF2, CYTBR2 and BHR1. Amplification of the smaller fragment is a positive ID for *B. hortorum*. The larger fragment acts as an internal positive control and amplification of this fragment without the smaller fragment (05-07) is a negative ID for *B. hortorum*.

Microsatellite genotyping

Five *B. ruderatus* individuals, from five different sampling locations, were used to test fifteen microsatellite loci for amplification and their degree of polymorphism. Of the fifteen loci tested, five were monomorphic (BT02, BT05, BL10, BL13 and BL15) and two did not amplify well (BL08, BT30) (Funk *et al.* 2006). The remaining eight loci amplified well and were polymorphic, therefore individuals were then genotyped at the eight usable microsatellite loci: BT01, BT08, BT10, BT13, BT26, BL11, BTERN01 and BTERN02 (Funk *et al.* 2006). Amplification took place in 15µl PCR reactions containing 0.5µl DNA extract and 14.5µl master mix that consisted of 1.5µl 10X NH_4 Buffer, 0.6µl 50mM $MgCl_2$, 0.6µl 2mM dNTPs, 0.5µl of each 10mM primer (the forward primer for each locus was fluorescently labelled with either 6-FAM, VIC, NED or PET (Applied Biosystems)), 0.12µl of 5 U/µl BioTaq™ (Bioline) and 10.68µl ddH_2O. The PCR protocol involved an initial denaturing step at 95°C for 12 minutes, followed by 10 cycles of denaturing at 94°C for 15 sec, annealing at (loci dependent) (Table 3.1) Ta°C for 30 sec and extension at 72°C for 30 sec, followed by 30 cycles of denaturing at 89°C for 15 sec, annealing at (loci dependent) Ta°C for 30 sec, extension at 72°C for 30 sec, then a final extension of 72°C for 10 minutes. Samples were prepared for genotyping by combining 0.5µl of PCR product with 12µl HiDi Formamide and 0.3µl of internal size standard 500 LIZ® (GeneScan™), then denatured at 95°C for four minutes. The PCR products were genotyped on an ABI 3130*xl* capillary sequencer. Genotypes were determined manually using GENEMARKER ver. 2.2.0.

Table 3.1: Annealing temperature and allele size range for each locus.

Locus	*Ta (°C)*	*Size range (bp)*
BT01	53	147-161
BT08	52	137-147
BT10	53	138-160
BT13	51	150-166
BT26	52	95-125
BL11	48	124-146
BTERN01	51	100-124
BTERN02	52	177-221

Data Analysis

The program COLONY v2.0 (Jones & Wang 2010) was used for detecting siblings as a comparison of programs that infer family structure found COLONY to be the most reliable for bumble bees (Lepias *et al.* 2010). The program constructs 'sibships', which in essence assigns individuals to distinct 'colonies' within each population. The software was run with the settings for haplodiploid species with 10 runs per population. A single representative was chosen at random from each colony for subsequent analyses, preventing the observed pattern of population structure from being obscured by family structure by removing genetic pseudo-replication. The COLONY program uses maximum likelihood methods to assign sibling-to-sibling relationships and takes into account genotyping error. Genotyping error was set at 2% (allele drop out 0.5% and other 1.5%).

MICRO-CHECKER v 2.2.3 (Van Oosterhout *et al.* 2004) was used to test for the presence of null alleles. ARLEQUIN v 3.5.1.2 (Excoffier & Lischer 2010) was used to test for Linkage-Disequilibrium between pairs of loci, and for deviations from Hardy-Weinburg Equilibrium (HWE) for each locus. Standard measures of genetic diversity, expected heterozygosity (H_E) and allelic richness (AR) were calculated using ARLEQUIN. Genetic differentiation (F_{ST}) and the inbreeding coefficient (F_{IS}) (Weir and Cockerham 1984) were determined using an AMOVA with 1000 permutations implemented in ARLEQUIN. F_{ST} is a measure of genetic differentiation between populations that is based upon the variance in allele frequencies among populations, ranging between 0 (no differentiation or panmixia) and 1 (total differentiation) (Holsinger & Weir 2009; Meirmans & Hedrick 2011). The analogue R_{ST} (Slatkin 1995), specifically designed for use with microsatellites, is only reliable when the stepwise mutation model is followed (Balloux & Lugon-Moulin 2002) and was not used because several loci did not have allele frequencies that followed a normal distribution (a normal

distribution of allele frequencies is expected under the stepwise mutation model). Pairwise genetic differentiation between populations were estimated using F_{ST} (Weir and Cockerham 1984) and tested for significance with 1000 permutations in ARLEQUIN. All F_{ST} values were then corrected for within population variation by calculating F'_{ST} (actual F_{ST} divided by maximum F_{ST}) following Meirmans & Hedrick (2011) in GENODIVE v 2.0b23 (Meirmans & van Tienderen 2004). False Discovery Rate (FDR) correction for multiple tests was used to minimize type I errors whilst retaining statistical power when compared to the Bonferroni correction method (Narrum 2006).

A Mantel test (Legendre & Legendre 1998) was implemented in GENODIVE with 1000 permutations to test for the pattern of isolation by distance (IBD), assessing the correlation between physical distance and genetic differentiation between populations (using pairwise F_{ST}). A significant positive relationship between F_{ST} and physical distance is most likely to occur if populations are separated by a homogeneous landscape (with respect to landscape features that can influence the dispersal of bumble bees), and the level of genetic differentiation is, therefore, driven mostly by the physical distance individuals need to move between populations. Therefore, to assess the potential influence of landscape factors on gene flow, CIRCUITSCAPE was used to compute pairwise resistance between sites using circuit theory (McRae 2006). This method treats the landscape matrix like an electronic circuit, where each cell in a raster map that represents the landscape is given an electric resistance value. The ease at which individuals can disperse between sites is then modelled as the way the electric current flows between points in the circuit (McRae 2006).

Data for the models were obtained from two maps, NZLRI Vegetation Cover and NZDEM South Island 25 metre (Landcare Research NZ Ltd), both downloaded from the LRIS portal (http://lris.scinfo.org.nz). Using ArcGIS v 9, a resistance value was assigned to each cell in the raster based upon the landscape data and then models were exported in the ASCII file format. A null model was created to test for IBD by assigning a value of 1 to all cells in the grid. This method is used for assessing IBD when using resistance maps instead of physical distance between sites as it accounts for any influence the grid boundary has on the resistance models (Koen *et al.* 2010; Amos *et al.* 2012). The first model was created to assess the influence of elevation. A resistance value of 1 + (altitude/100) was assigned to each cell as used by Goulson *et al*. (2011). Whilst *Bombus* species are considered to be alpine adapted, there is some evidence that the Alps in Europe have acted as a barrier to gene flow for *B. pascuorum* (Widmer & Schmid-Hempel 1999). The second model was created to assess the influence of habitat type. The 54 categories of land cover in the NZLRI map were condensed into two categories: poor habitat (included all native vegetation and urban areas) was assigned a value of 20 and good habitat (included cropland, non-native grassland, non-native

scrub, river and lake areas) was assigned a value of 1. Assignment of land cover was based upon habitat preference for *B. ruderatus* (Goulson & Hanley 2004; Goulson *et al.* 2006). As pointed out by Goulson *et al.* (2011), the actual resistance values used are arbitrary; they are conceptual values that are compared to one another within each model run in CIRCUITSCAPE. The maps were then analysed in CIRCUITSCAPE using the pairwise iteration model with an eight neighbour connection scheme. As the hypotheses for the relationships between genetic differentiation and resistance to gene flow are in terms of distances (Legendre & Fortin 2010), Mantel tests and partial Mantel tests (Smouse *et al.* 1986) were then implemented in GENODIVE using 1000 permutations, to test for isolation by resistance (IBR) for each model whilst controlling for competing models (Cushman *et al.* 2006; McRae & Beier 2007). To obtain the correct type I error, the significance testing for partial Mantel tests was conducted following Legendre (2000) using permutation method 2 and corrected for multiple tests using the FDR method. Scatter plots were created in R (R Development Core Team 2008) using the package "ggplot2".

STRUCTURE v 2.3.4 (Pritchard *et al*. 2000) was used to determine population structure with Bayesian cluster analysis. The Admixture model (Falush *et al*. 2003) was used and the analysis was run with prior location information to assist the clustering when population structure is weak, as recommended by Hubisz *et al.* (2009). This was run with the number of clusters (K) varying from 1 to 7, as 7 is the number of sampling sites, with 15 runs for each K value, the burn in set to 10,000 with 50,000 Markov Chain Monte Carlo repetitions. An additional analysis was conducted using prior population information to detect recent migrants between predefined populations. STRUCTURE HARVESTER (Earl & vonHoldt 2012) was used to visualize the results and determine the number of populations using both mean likelihood (L'(K)) and Delta K (Evanno *et al.* 2005) methods.

Results

Genetic diversity, HWE and Linkage Disequilibrium

A total of 224 individuals were genotyped for eight loci. Following removal of sibling workers the remaining analyses were conducted with a total of 150 individuals. One locus (BT13) was significantly out of HWE at four out of the seven sites and was therefore excluded from further analyses. The remaining loci did not show any significant deviation from HWE across any of the sites (BT10 & BL11) or showed significant deviation from HWE at only one location (BT01, BT08, BT26, BTERN01, BTERN02). The remaining loci also showed no significant linkage disequilibrium across multiple sites, therefore all of these markers were retained. Micro-checker detected the potential

presence of a null allele in 3 out of 49 tests (Queenstown BT08, BL11 and Alexandra BTERN01). Overall levels of genetic diversity were moderate with average allelic richness at 4.88 (±1.65), ranging from 3.14 (±1.06) to 5.86 (±2.27) and average H_E across sites at 0.633 (±0.126), ranging from 0.656 (±0.122) to 0.562 (±0.120). The overall F_{IS} value was low but significant (F_{IS}=0.07, P<0.001). Two of the populations showed significant levels of inbreeding, Queenstown (F_{IS}=0.25, P=0.007) and Alexandra (F_{IS}=0.10, P=0.036) (Table 3.2).

Table 3.2: Standard measures of genetic diversity for each population; N (sample size), $N_{(colonies)}$ (sample size used for analyses, the number of colonies at each site), H_E (expected heterozygosity), SD (standard deviation), H_O (Observed heterozygosity), AR (allelic richness) and F_{IS} (inbreeding coefficient). Significant values are in bold.

Population	*N*	*N (colonies)*	*H_E*	*SD*	*H_O*	*AR*	*SD*	*F_{IS}*	*P (F_{IS})*
Lake Tekapo	57	37	0.655	±0.146	0.629	5.86	±1.13	0.039	0.201
Queenstown	17	10	0.562	±0.120	0.429	3.14	±0.69	**0.247**	**0.007**
Nelson	36	19	0.616	±0.097	0.556	4.57	±1.50	0.099	0.063
Wanaka	26	20	0.660	±0.098	0.643	5.00	±1.07	0.027	0.354
Alexandra	28	21	0.663	±0.125	0.599	5.43	±1.72	**0.099**	**0.036**
Waitaki River	29	20	0.622	±0.170	0.586	5.00	±1.07	0.059	0.161
Twizel	31	23	0.656	±0.122	0.617	5.14	±2.12	0.060	0.156

Genetic structuring of populations

The pairwise F_{ST} comparing samples collected in January and March at Lake Tekapo indicated that there was no differentiation between these sampling periods (F_{ST}<0.001, P=0.7) and so they were grouped for further analysis. Overall genetic differentiation between sites was relatively low but significant (F_{ST}=0.035, F'_{ST}= 0.096, P<0.0001). Pairwise F_{ST} (F'_{ST}) values ranged from <0.001 (< 0.001) to 0.137 (0.314) and 13 out of 21 of these comparisons were significant after adjusting the α level for multiple tests (Table 3.3). The Nelson population showed moderate levels of pairwise differentiation and was significantly differentiated from all of the other sites (F_{ST}/F'_{ST} from 0.069/0.159 to 0.136/0.314). Out of the remaining populations, Alexandra was not significantly differentiated from any of the other sites except for Twizel (F_{ST}/F'_{ST}= 0.03/0.088, P=0.002). The Queenstown and Wanaka sites were significantly differentiated from Waitaki River, Twizel and Lake Tekapo (Table 3.3).

Table 3.3: Population pairwise F_{ST}/F'_{ST} value below diagonal, P values for F_{ST} above diagonal. P values were corrected using the False Discovery Rate (FDR) method for multiple tests and significant values are in bold.

	Lake Tekapo	*Queenstown*	*Nelson*	*Wanaka*	*Alexandra*	*Waitaki River*	*Twizel*
Lake Tekapo	-	**0.003**	**< 0.001**	**<0.001**	0.04	0.204	0.129
Queens-town	**0.043/ 0.107**	-	**< 0.001**	0.039	0.025	**0.002**	**< 0.001**
Nelson	**0.062/ 0.162**	**0.136/ 0.314**	-	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
Wanaka	**0.031/ 0.093**	0.032/ 0.086	**0.090/ 0.234**	-	0.436	**0.011**	**< 0.001**
Alexandra	0.014/ 0.043	0.041/ 0.104	**0.069/ 0.180**	0.002/ 0.006	-	0.496	**0.002**
Waitaki River	0.006/ 0.023	**0.056/ 0.115**	**0.069/ 0.159**	**0.024/ 0.059**	<0.001	-	0.155
Twizel	0.008/ 0.014	**0.074/ 0.172**	**0.069/ 0.178**	**0.042/ 0.132**	**0.029/ 0.089**	0.009/ 0.032	-

The Mantel test showed significant support for isolation by distance (IBD) (r=0.8, P=0.005) between pairwise F_{ST} and physical distance between populations (Figure 3.2). However, this relationship was no longer significant (r=0.34, P=0.08) when the Nelson population was removed from the analysis (Figure 3.3a). The resistance models were run without Nelson to assess the effect of landscape factors on gene flow in the absence of a significant impact of physical distance. Partial Mantel tests were used to test for a correlation between pairwise F_{ST} and each model whilst controlling for the null model to account for the effect of physical distance between sites.

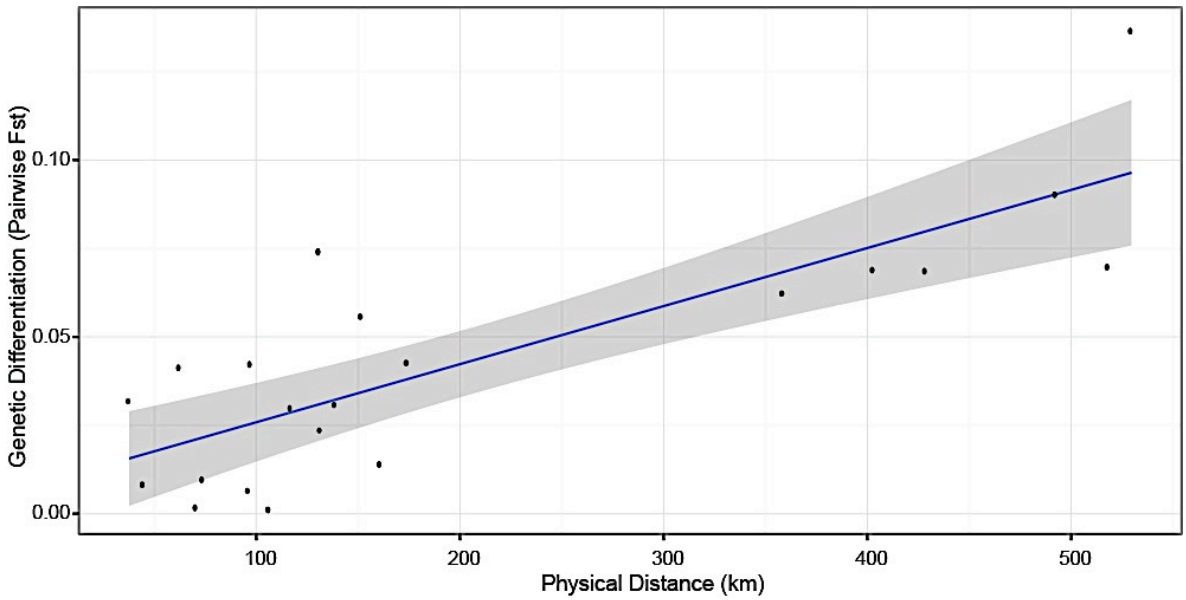

Figure 3.2: Mantel correlation between pairwise physical distance (km) and pairwise genetic distance (F_{ST}). Mantel's r = 0.81, P = 0.005. The shaded area is the standard error.

The model testing the influence of elevation was significant and there was an improvement in the partial Mantel r-value, compared to the relationship between physical distance and pairwise F_{ST} (r = 0.635, P = 0.018; Figure 3.3b). The model testing the influence of habitat was also significant and the partial Mantel r-value was greater than for the elevation model (r = 0.873, P = 0.001; Figure 3.3c). To compare the relative influence of elevation and habitat type on gene flow, partial Mantel tests including both elevation and habitat were used to test for a correlation while controlling for the influence of the competing model. The partial Mantel test between elevation and F_{ST} was marginally significant when controlling for habitat, and this correlation was now negative (r = -0.497, P = 0.048). The weak partial correlation between elevation-mediated resistance and F_{ST} when controlling for habitat-mediated resistance may be negative because there was a decrease in pairwise F_{ST} with increasing elevation within groups when the data were grouped on the basis of low, medium and high pairwise habitat resistance, however the overall relationship between elevation and F_{ST} was positive (Figure 3.4). The partial Mantel test between habitat and F_{ST} remained significant when controlling for elevation and there was still a strong positive correlation (r = 0.815, P = 0.001). This indicates that habitat is an important factor mediating dispersal and gene flow between populations of *B. ruderatus* in the central South Island.

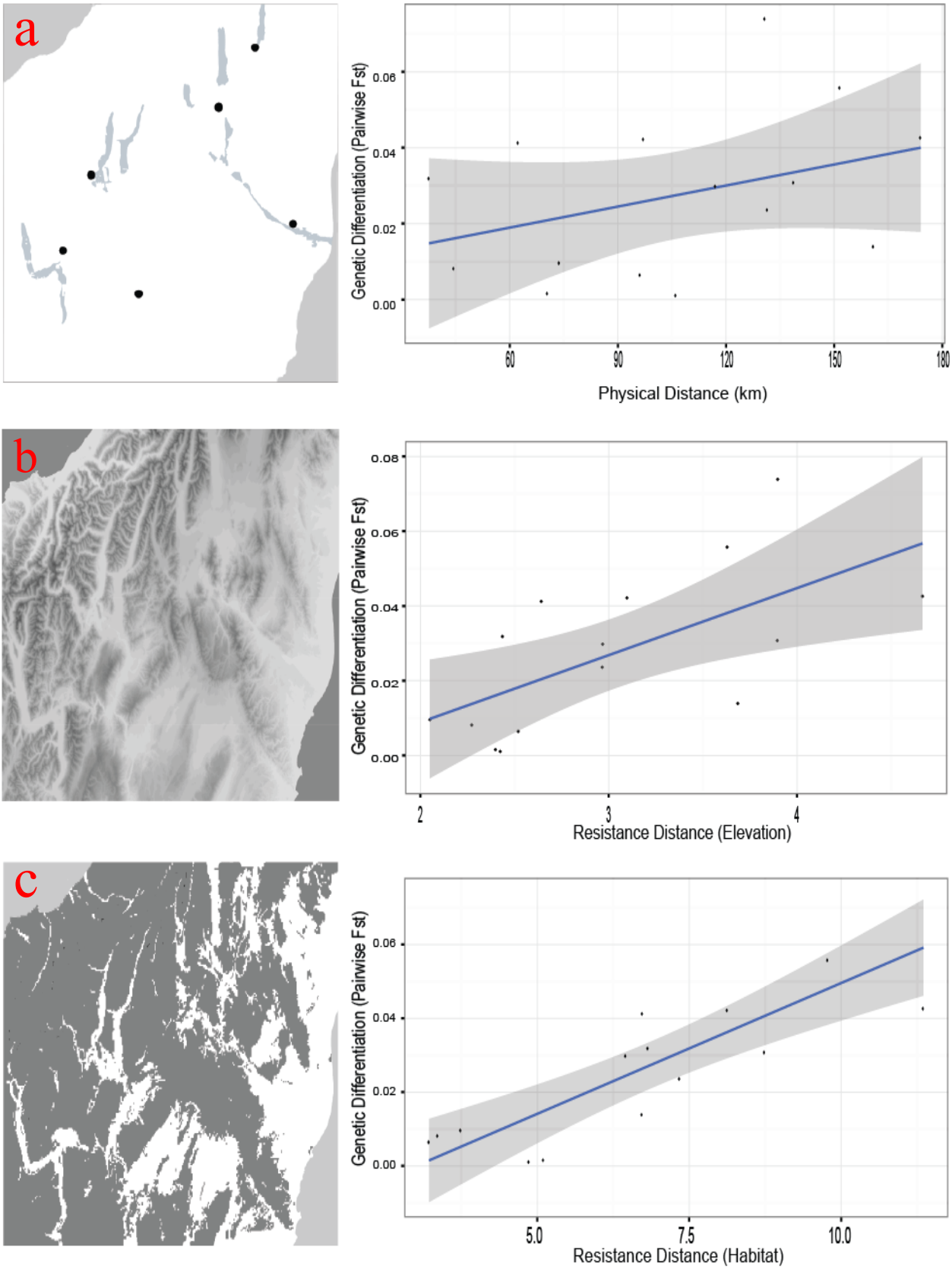

Figure 3.3: Mantel correlations between genetic differentiation (pairwise F_{ST}) and; a - physical distance (km) (sites are shown as black circles, lakes and rivers are shown for reference only and did not impact on movement of individuals), b - resistance (elevation) and c - resistance (habitat), excluding samples from Nelson. The areas of ocean were impassable on all maps and for maps b and c, darker colour indicates increased resistance. Shaded area is the standard error.

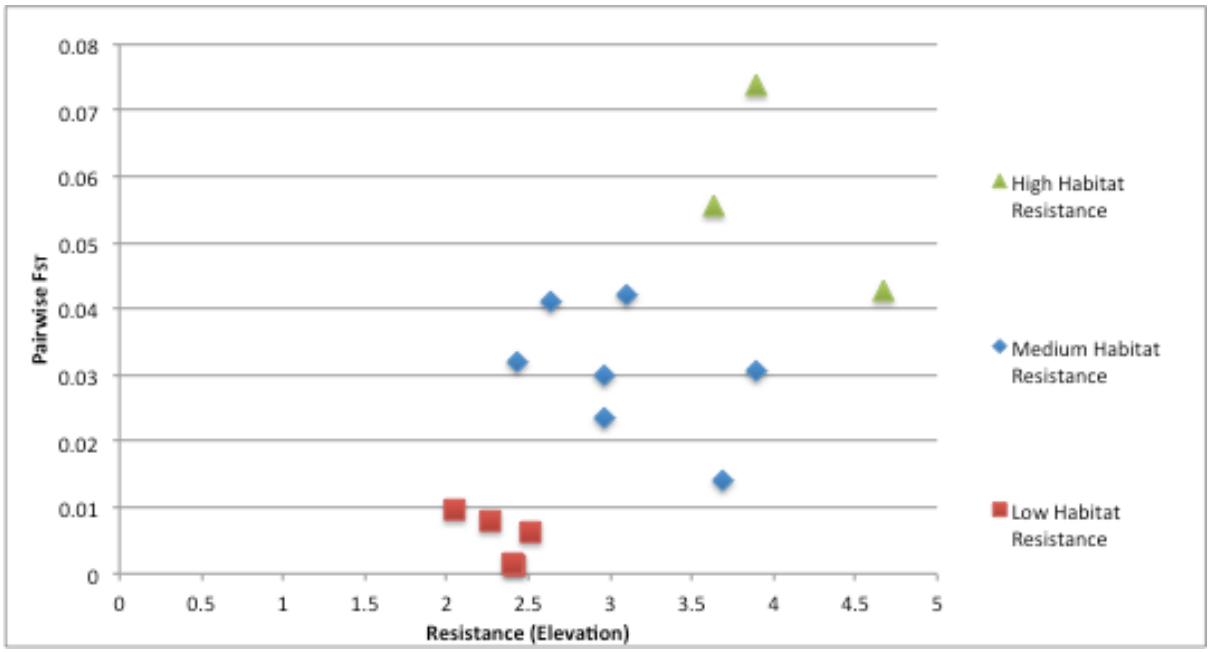

Figure 3.4: Scatterplot of the same data from Figure 3.3b, genetic differentiation (pairwise F_{ST}) against pairwise resistance (elevation), with data grouped by habitat resistance. This shows that although the overall correlation between elevation resistance and F_{ST} is positive, there is a negative trend within the habitat resistance groups. This is the reason why the r-value for the Mantel test between elevation resistance and F_{ST} was positive but the r-value for the partial Mantel test between elevation resistance and F_{ST} was negative when controlling for habitat resistance.

The cluster analysis in STRUCTURE suggests that the most likely number of populations was K = 2 using delta K and K = 3 based upon the highest log likelihood (Figure 3.5a). The bar plot of the K = 3 clustering supports this, showing distinct clusters for Nelson; Queenstown; and Twizel, with a gradual change in composition from Queenstown to Twizel at the remaining sites (Figure 3.6a). To further investigate the structure of the populations in the central South Island, Nelson was removed (as a clearly distinct population that is isolated by distance) and the analysis was run again. This was to allow STRUCTURE to better define the clustering without the large difference of the Nelson samples obscuring the more subtle differences between the remaining populations. The highest log likelihood was for K = 2 although this was not much greater than the log likelihood for K = 1 (Figure 3.5b). The delta K method also suggests K = 2, however this method is not useful when determining if the number of groups is either one or two as it cannot estimate a value for K = 1 (Evanno *et al.* 2005). The bar plot for K = 2 again suggests that there are two distinct populations dividing Queenstown and Wanaka from Twizel, Waitaki River and Lake Tekapo with Alexandra intermediate (Figure 3.6b). The analysis with prior population information was unable to detect any recent first or second-generation migrants.

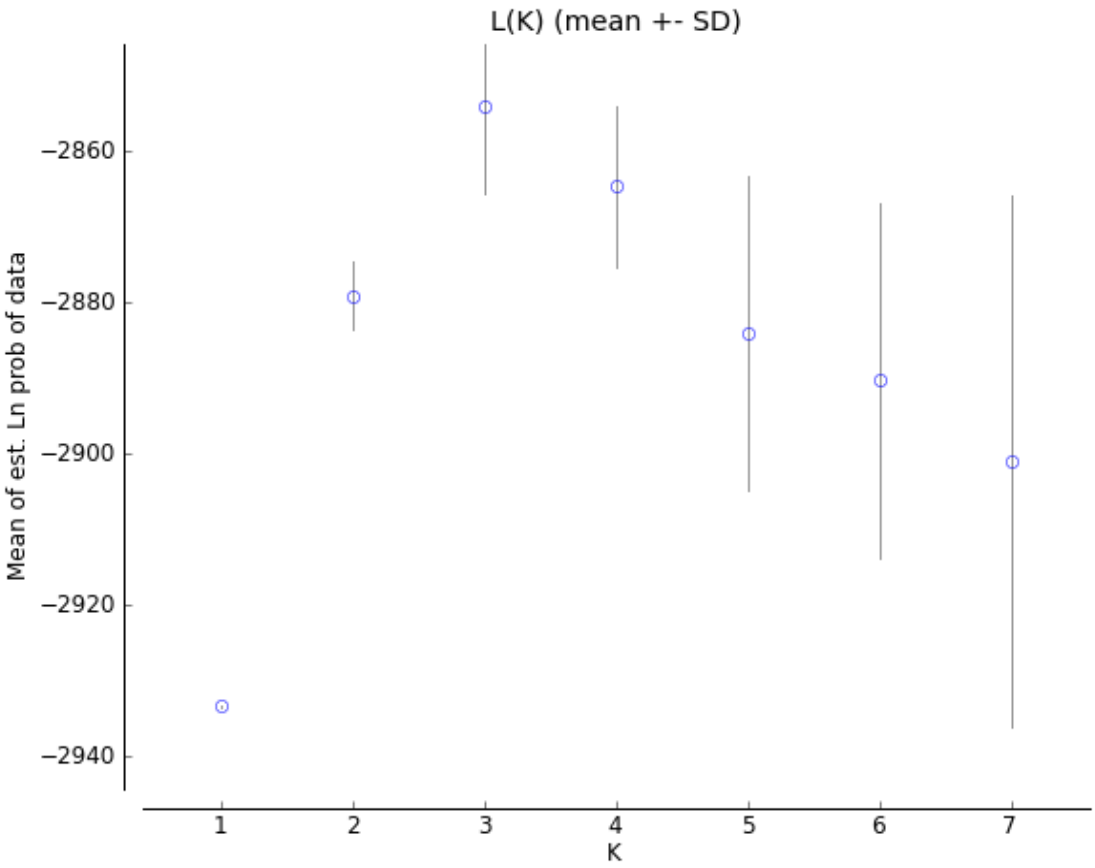

Figure 3.5a: Cluster analysis of microsatellite data using STRUCTURE. The most likely number of groups (K) will have the greatest log likelihood value (est. Ln). The analysis suggests there are 3 genetically distinct groups. Figure was produced using STRUCTURE HARVESTER (Earl & vonHoldt 2012).

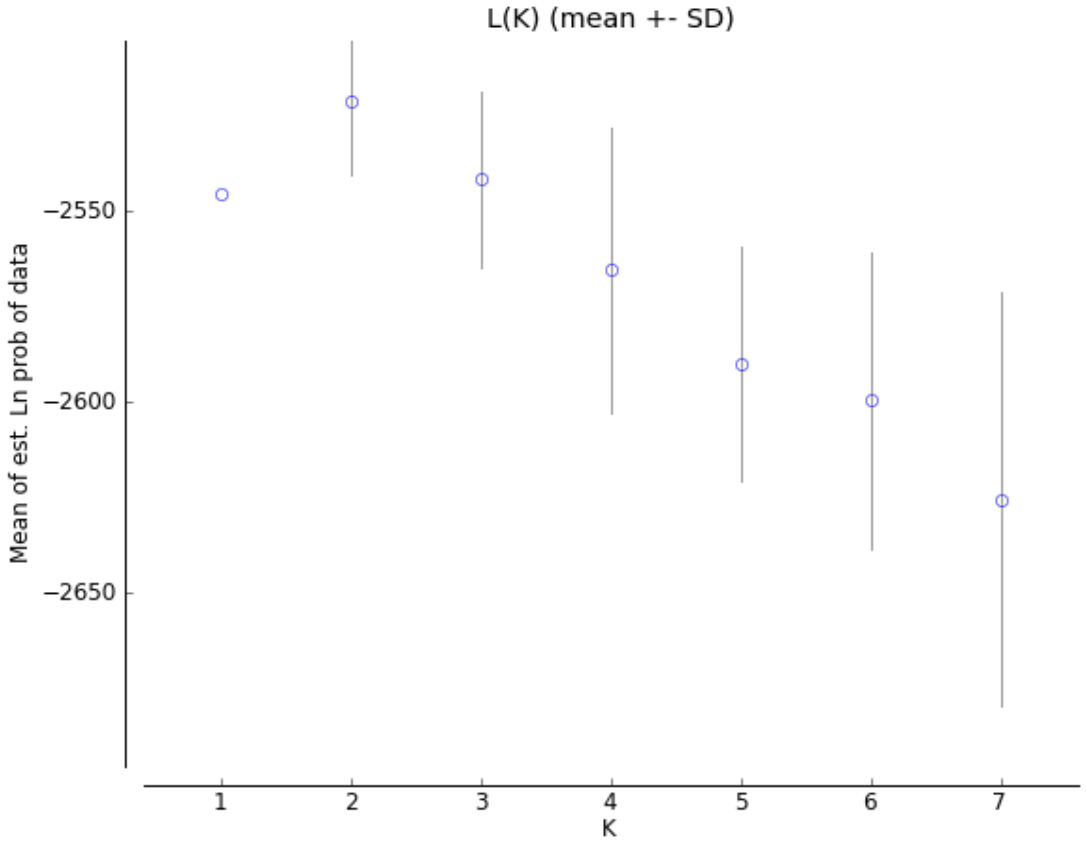

Figure 3.5b: Cluster analysis of microsatellite data with Nelson excluded using STRUCTURE. The most likely number of groups (K) will have the greatest log likelihood value (est. Ln). The analysis suggests there are 2 genetically distinct groups, although the est. Ln for K =1 is similar. Figure was produced using STRUCTURE HARVESTER (Earl & vonHoldt 2012).

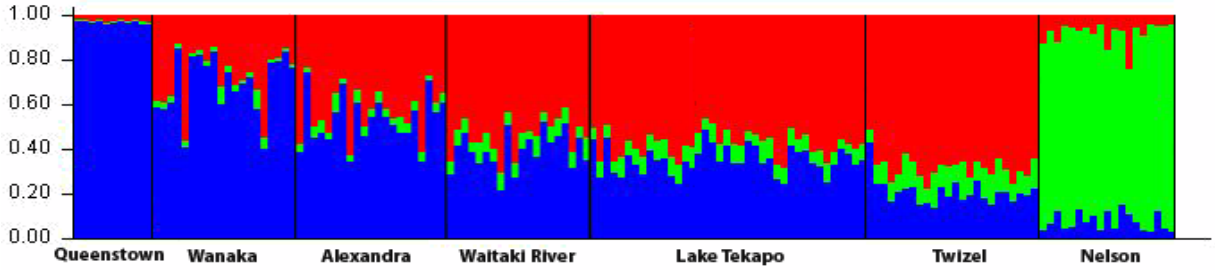

Figure 3.6a: STRUCTURE bar plot. There are three groups; Nelson is distinct, Queenstown with Wanaka, and Twizel with Lake Tekapo. The remaining populations are intermediate with a gradual change in composition from Queenstown to Twizel.

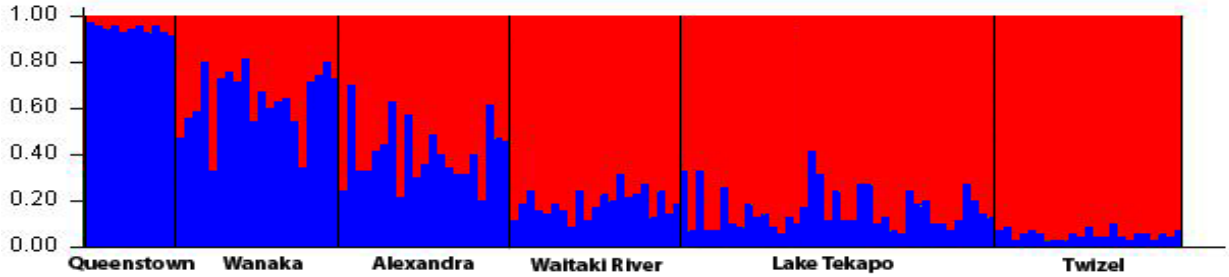

Figure 3.6b: STRUCTURE bar plot. Nelson was excluded from this analysis. There are two groups; Queenstown with Wanaka and Twizel with Lake Tekapo and Waitaki River. Alexandra is intermediate.

Discussion

There was significant genetic differentiation between populations of *B. ruderatus* in the South Island. Although the overall F_{ST} was low, it was significantly greater than zero (F_{ST} = 0.035, F'_{ST} = 0.096, P<0.0001), and the pattern of pairwise F_{ST} values and the results from the cluster analysis both support the presence of distinct genetic groups. The first cluster analysis using STRUCTURE placed the samples from Nelson in a cluster distinct from the other samples. Pairwise F_{ST} values also indicated that Nelson is isolated from the other populations with the significant isolation by distance relationship suggesting that this is due to the comparatively large geographic distance between Nelson and the other sites. Of the remaining populations, the second cluster analysis found that there are most likely two groups, one consisting of individuals from Queenstown and Wanaka, and the other with individuals from the Twizel, Lake Tekapo and Waitaki River populations. This suggests that there are relatively low levels of gene flow between these two groups compared to the levels of gene flow within them. This pattern is likely to be the outcome of the range expansion of *B. ruderatus* through this area, combined with the effects of gene flow, drift and selection up to the present day. The population at Alexandra appears to be intermediate to both groups, with very low and non-significant pairwise F_{ST} values between Alexandra and both the Waitaki River and Wanaka populations suggesting that there may be relatively high levels of gene flow between these populations.

The correlation between physical distance and pairwise F_{ST} when Nelson was excluded was non-significant and this suggested that the observed pattern of significant genetic differentiation between these remaining populations is likely due to landscape factors, rather than physical distance, limiting gene flow. Evidence for the possibility of landscape factors influencing gene flow can be seen when the influence of elevation or habitat on dispersal is included, resulting in a now significant Mantel test and improved model fit, even when controlling for the effect of physical distance using the null model (Figure 3.3). The partial Mantel tests suggest that habitat type and elevation are correlated (correlation coefficient = 0.89), which is not surprising given that vegetation at both the low and high-alpine zones is characterised by native grassland leading into sparse cover of high-alpine native vegetation (Mark *et al*. 2000). The results suggest that habitat is the better predictor, as the habitat model remains highly significant with a strong positive correlation when controlling for elevation, while the elevation model has a marginally significant negative partial correlation when controlling for habitat.

The pattern of genetic structure in *B. ruderatus* differs from that found in *B. terrestris* in the South Island. Whilst there appears to be restricted gene flow between some populations of *B. ruderatus,* no significant differentiation was detected between populations of *B. terrestris* (Schmid-Hempel *et al.* 2007). Levels of gene flow between *B. terrestris* populations are expected to be greater, as the distribution of *B. terrestris* in the South Island is more continuous than *B. ruderatus* (Chapter 2). A comparison of the genetic structure of *B. ruderatus* and *B. hortorum* in New Zealand would be interesting given these closely related species have very similar niches and the reason for the decline of *B. ruderatus* in the UK whilst *B. hortorum* remains common is not fully understood. Goulson *et al.* (2011) found that despite being one of the 6 most common *Bombus* species in Europe, populations of *B. hortorum* were unexpectedly genetically differentiated in comparison with other less common *Bombus* species in their study area in the Western Isles of Scotland. These populations also had relatively high levels of genetic diversity, suggesting that they have persisted as relatively large and stable populations for some time despite a lack of gene flow.

Bombus species that have undergone declines in their native ranges have been generally characterized as having lower levels of genetic diversity and greater population structuring than non-declining species (Goulson *et al*. 2008; Cameron *et al.* 2011). Whilst *B. ruderatus* appears to have undergone a decline in its distribution's range in the South Island since 1995 (Chapter 2), overall levels of genetic diversity in *B. ruderatus* are comparable to non-declining *Bombus* species (Table 3.4). This suggests that although the distribution of *B. ruderatus* may have declined recently, most remaining populations appear to be stable, or it is still too early to detect any appreciable decrease in diversity due to drift. Levels of genetic diversity comparable to those of species in their native ranges (Table 3.4) suggest that a relatively large number of *B. ruderatus* was initially introduced, or that founders were collected from several source populations in the UK. It is also possible that *B. ruderatus* was represented in both the 1885 and 1906 introduction events.

Lye *et al.* (2011) consider *B. ruderatus* in New Zealand to be moderately differentiated from its source population in the UK (F_{ST} = 0.07, P<0.001, calculated over 8 microsatellite loci), however they failed to detect a significant reduction in allelic richness or Nei's unbiased measure of gene diversity between *B. ruderatus* from a source population in the UK and Twizel in New Zealand. This lack of difference in genetic diversity was thought to be due to the now reduced diversity in the modern UK population following the rapid decline of *B. ruderatus,* with levels in New Zealand likely representing only part of the diversity in the source population when the introduced individuals were taken (Lye *et al*. 2011). The physical distance between Twizel and the original introduction area is approximately 200 km, so the number of founders predicted by Lye *et al.* (2011) from data

Table 3.4: Indices of genetic diversity (H_E = expected heterozygosity, AR = allelic richness) in non-declining *Bombus* species in their native ranges. These studies all used microsatellite markers. Data from this study is given for reference. Standard deviations are given where available

Species	***B. ruderatus***	***B. terrestris***	***B. hortorum***	***B. pascourum***	***B. bifarius***	***B. impatiens***
Study Location	New Zealand (SI)	Europe	Scotland	Southern England	USA	USA
H_E	0.633 (±0.126)	0.41 (±0.09) –0.65 (±0.07)	0.74	0.563 (± 0.009)	0.771 (±0.042)	0.687 (±0.018)
AR	4.88 (±1.65)	3.8 (±0.5) – 6.5 (±1.4)	4.2	5.49 (± 0.16)	5.436 (±0.46)	4.737 (±0.19)
Source	This research	Estoup *et al.* (1996)	Goulson *et al.* (2011)	Darvill *et al.* (2006)	Lozier *et al.* (2011)	Lozier *et al.* (2011)

collected in Twizel could have been underestimated due to the effect of additional bottlenecks between the initial introduction(s) and spread. Therefore an explanation as to why levels of genetic diversity observed in New Zealand are not significantly lower than that of the source population (as is also the case for *B. terrestris*) may be because the number of individuals introduced may actually have been closer to the number Lye *et al*. (2011) predicted for *B. terrestris* than for *B. ruderatus* (58 compared to 14).

The Queenstown population has relatively low levels of genetic diversity (Table 3.2). Pairwise F_{ST} values and cluster analysis both indicate that Queenstown bumble bees are relatively genetically isolated from the populations further north, with any gene flow likely coming from the Wanaka population as they are clustered together. The relatively high pairwise F_{ST} values between Queenstown and Alexandra (F_{ST}/F'_{ST} = 0.041/0.104, P = 0.025), and Queenstown and Wanaka (F_{ST}/F'_{ST} = 0.032/0.086, P = 0.039) were non-significant after correcting the P value for multiple comparisons, however this may be to be due to the small sample size after removing siblings (n = 10) for Queenstown. Significant Pairwise F_{ST} values between Queenstown and the remaining populations despite this low sample size suggest that these differences are substantial (Table 3.3). Pairwise F_{ST} values between the other central South Island sites that group together in the cluster analyses are comparatively much lower (Table 3.3). Based on the resistance map (Figure 3.3c), it appears gene flow is limited because dispersal of bumble bees from the north into the Queenstown area is only facilitated by the corridor of habitat along the Karawau River. This in

combination with the detection of a significant inbreeding coefficient (F_{IS} = 0.25, P = 0.007) suggests that the Queenstown population may be less stable than the other populations.

Inbreeding and associated inbreeding depression can seriously influence the persistence of wild populations (Frankham 1995; Keller & Waller 2002, O'Grady *et al.* 2006). Social hymenoptera may be particularly vulnerable to inbreeding and genetic factors influencing population persistence due to low effective population sizes (Chapman & Bourke 2001; Packer & Owen 2001). The effect of inbreeding on haplodiploid species is expected to be additionally detrimental because homozygosity at the sex-determining locus (or loci) causes fertilized eggs to develop into sterile diploid males instead of workers or queens (Cook & Crozier 1995; Packer & Owen 2001). Sterile diploid male production was found to significantly reduce the fitness of *B. terrestris* colonies (Whitehorn *et al.* 2009).

In summary, there is genetic structuring between populations of *B. ruderatus* in the South Island. The presence of groups in the cluster analysis in combination with the pattern of pairwise F_{ST} values suggests that there is greater connectivity between populations within each group than between populations in different groups. Alexandra is intermediate between the Queenstown-Wanaka group and the Lake Tekapo, Twizel and Waitaki River group. The resistance models suggest that suitable habitat is the main factor influencing the connectivity between populations at the sub-200 km scale. The observed pattern of differentiation is likely to be a result of the combination of gene flow and genetic drift as well as the historical signature from colonisation and initial range expansion. This pattern provides an expectation of the levels of differentiation expected due to genetic drift and the importance of this is addressed in Chapter 4. A clear understanding of the genetic structure and connectivity between populations in the central South Island is essential for interpreting the influence of gene flow on the maintenance of body colour polymorphism in these populations (discussed in Chapter 5).

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CHAPTER 4: Maintenance of body colour polymorphism in *Bombus ruderatus* in the South Island, New Zealand.

Bombus ruderatus is polymorphic for body colour, showing variation in the degree of melanism in workers, queens and males (Donovan 2007). As outlined in Chapter 1, several possible processes could maintain colour polymorphism in *B. ruderatus* in New Zealand. In Chapter 2, significant differences in both climatic conditions and the relative abundances of bumble bee species between sampling locations were described, which relate to three potential selective pressures that could be maintaining colour polymorphism. Genetic structuring of *B. ruderatus* populations in the South Island was then shown in Chapter 3, so that the impact of selectively neutral processes on body colour polymorphism could be evaluated. This chapter will combine environmental, genetic and body colour data to assess the relative roles of selection, selectively neutral processes and phenotypic plasticity in the maintenance of colour polymorphism in *B. ruderatus* in the South Island of New Zealand.

Selection pressure for colour polymorphism

Body colour is expected to influence the fitness of individuals, with melanism known to influence several physiological processes and biotic interactions in insects (True 2003). Selection for or against melanism is therefore one mechanism that could be important in the maintenance of colour polymorphism in *B. ruderatus*. The thermal melanism hypothesis suggests that ectotherms, such as bumble bees, will benefit from dark colouration in colder environments due to faster rates of heating and cooling, resulting in relatively higher body temperatures at the same levels of solar radiation (True 2003; Clusella-Trullas *et al.* 2007). Evidence for thermal melanism has been detected in reptiles (Clusella-Trullas *et al.* 2008; Castella *et al.* 2013), molluscs (Phifer-Rixey *et al.* 2008) and in insects: Hemiptera (Punzalan *et al.* 2008a), Coleoptera (Brakefield & Wilmer 1985; de Jong *et al.* 1996), Lepidoptera (Goulson 1994; Ellers & Boggs 2004; Karl *et al.* 2009) and Orthoptera (Forsman 1999; Ahnesjö & Forsman 2006; Harris *et al.* 2013). However, not all studies have reported an effect of body colour on thermal properties (Bots *et al.* 2008; Umbers *et al.* 2013) and some found that morphs could account for the affect of melanism on thermoregulation with behavioural or physiological adaptations (Forsman *et al.* 2002; Ahnesjö & Forsman 2006; Sandre *et al.* 2007; Forsman 2011). In bumble bees there is some support for the hypothesis that body colour influences thermoregulation (Pekkarinen 1979; Stiles 1979; Williams 2007). If thermal melanism is important for *B. ruderatus* in New Zealand, then an increase in the frequency of melanic forms is expected at sites with colder ambient temperatures.

Melanism has also been linked to desiccation tolerance in *Drosophila,* with darker individuals better able to retain moisture and as such have greater fitness than lighter coloured individuals in arid environments (Brisson *et al.* 2005; Parkash *et al.* 2008; Rajpurohit *et al.* 2008; Ramniwas *et al.* 2013). Brisson *et al.* (2005) found that darker pigmentation was significantly associated with open areas, with lighter coloured individuals more frequent in forested habitat. In New Zealand, *B. ruderatus* is found at greater abundance where the climate is warmer and drier (See Chapter 2). Lye *et al.* (2010) also found that *B. ruderatus* forages primarily in the middle of the day, when conditions are hottest and driest. If melanism has an important influence on tolerance to desiccation in *B. ruderatus* then the frequency of melanic morphs is expected to increase where conditions are more arid.

A visual mechanism may also influence colour polymorphism in *B. ruderatus* as selection on body colour could be influenced by predation, with aposematism and Müllerian mimicry thought to be important in the evolution of bumble bee colouration (Plowright & Owen 1980; Williams 2007; Hines & Williams 2012). Experiments have found that predators such as toads (Brower *et al.* 1960) and birds (Evans & Waldbauer 1982) visually recognised and avoided bumble bees after initial exposure. Bumble bees also effectively use auditory signals when disturbed to deter predators (Kirchner & Röschard 1999; Goulson 2003; Jablonski *et al.* 2013). In New Zealand, *B. hortorum* closely resembles the *B. ruderatus* morph with black and yellow-banded colouration (Chapter 1: Colour Plate 1) although it does not show variation in melanism. If Müllerian mimicry were operating between these species, then an increase in the frequency of the non-melanic forms of *B. ruderatus* would be expected in sites where the relative abundance of *B. hortorum* is greater.

Divergence through drift

Phenotypic divergence of populations could alternatively be the result of genetic drift (Lande 1976; Lynch & Hill 1986). Genetic drift is the random change in allele frequencies across all loci, both selectively neutral and functional (Freeman & Herron 2007). Phenotypic divergence can be the result of these random changes in allele frequencies when populations are sufficiently isolated from one another, when gene flow cannot cause allele frequencies to become more similar, and when the changes are not opposed by selection (Lande 1976; Lynch & Hill 1986). The expectation for selectively neutral additive traits is that quantitative loci will behave like selectively neutral loci (McKay & Latta 2002). When the strength of selection varies temporally or spatially, genetic drift can periodically have more influence on morph frequencies than selection (O'Hara 2005; Oxford 2005). Some comparisons between levels of differentiation at selectively neutral and colour loci for

fish (Leinonen *et al.* 2006), amphibians (Hoffman *et al.* 2006) and reptiles (Runemark *et al.* 2010) have found no difference between the genetic structure at these loci, suggesting that differences in colour morph frequencies between populations could be the result of drift. A similar result was found in the eastern European populations of the damselfly *Ischnura elegans,* although not in the more recently established populations in Spain where divergent selection appeared to be more influential (Sánchez-Guillén *et al.* 2011).

Genetic basis of colouration and phenotypic plasticity

It is important to establish whether variation in colour is discrete or continuous (Teasdale *et al.* 2013). This is because the genetic basis of discrete and continuous traits may differ considerably, with continuous variation often being condition dependent and under the influence of multiple loci (Teasdale *et al.* 2013). Melanism in *Drosophila* is a continuous trait that does appear to have a genetic basis under polygenic control (Wittkopp *et al.* 2003a; Wittkopp *et al.* 2003b; Pool & Aquadro 2007; Wittkopp & Beldade 2009). The colour patterns on some butterfly species wings are also linked to multiple loci (Wittkopp & Beldade 2009). Heritability (h^2) of body colour in insects has been estimated for some species, with $h^2 = 0.42$ for wing colouration in the wood tiger moth, *Parasemia plantaginis* (Nokelainen *et al.* 2013) and $h^2 = 0.61$ for melanism in the sand cricket, *Gryllus firmus* (Roff & Fairbairn 2013). In the case of bumble bees, the genetic basis for a discrete component (either black or red pile) of the colour pattern in *B. melanopygus* fits the pattern of Mendelian inheritance expected when controlled by a single biallelic locus (Owen & Plowright 1980; Owen *et al.* 2010). Given that *B. ruderatus* shows a range of intermediate forms (Donovan 2007), this suggests that a genetic basis for melanism for this species may be more complex.

Variation in insect body colour can also be the result of phenotypic plasticity (Protas & Patel 2008). An individual's phenotype is the result of a series of developmental processes that are shaped by both genes and the environment; as such the same genotype can result in multiple different phenotypes when development occurs under different environmental conditions (West-Eberhard 1989; Nijhout 1999; Nijhout 2003). There are many examples of the environment influencing colour development in Lepidoptera (Goulson 1994; Kemp & Jones 2001; Talloen *et al.* 2004; Kemp 2008; Canfield *et al.* 2009; for review see: Brakefield & Frankino 2009). Within the Hymenoptera, expression of melanism in the paper wasp, *Polistes dominulus,* was strongly influenced by rearing temperature (Green *et al.* (2012). Expression of melanism in *P. dominulus* is also condition-dependent based on diet (Tibbetts 2010). These wasps use facial markings as a signal to evaluate other wasps when establishing dominance, with better quality markings developing in larvae that

received more food (Tibbetts 2010). Condition-dependent expression of melanism in the ambush bug *Phymata americana* was also due to diet (Punzulan *et al.* 2008b). Even when body colour has a genetic basis, variation within genetically determined morphs could be the result of a plastic response during development (Michie *et al.* 2010; Michie *et al.* 2011). Temperature is unlikely to be a major factor in the development of body colour in bumble bees because bumble bee queens actively incubate their developing brood and the temperature within their insulated colonies is regulated by workers (Heinrich 1979; Goulson 2003). However, diet may have an important influence as the pigment responsible for yellow colouration in bumble bees may be derived from pollen (Hines 2008) and considerable size polymorphism in bumble bee workers is linked to diet (Goulson 2003; Couvillon & Dornhaus 2009; Couvillon *et al.* 2010).

Assessing colour polymorphism in bumble bees

Up until recently, previous studies on bumble bee colouration have relied on observer-based scoring of colour templates (for example see Williams 2007; Hines & Williams 2012; for exceptions see Stelzer *et al.* 2010; Lozier *et al.* 2013). However, it is argued that studies of animal colouration should avoid using methods that rely on the subjective scoring of colour patterns by a human observer (Endler 1990). Spectrophotometry is an objective alternative to observer-based scoring that measures the reflectance of light across wavelengths in the visible spectrum for both humans and other animals (Endler 1990; Zuk & Decruyenaere 1994). However using spectrometers to effectively characterise the colour patterns over the entire surface of interest requires many point samples for each individual, which can be very time consuming (Stevens *et al.* 2007; Pike 2011). An alternative is to measure colour values from images taken using digital photography, which is comparatively much faster and can capture information for the entire surface that is photographed (Stevens *et al.* 2007; Bergman & Beehner 2008; Pike 2011).

My first aim was to objectively measure the body colour of *Bombus ruderatus* individuals collected from seven study sites. This was achieved using the digital photography method mentioned above. When colour was determined for each individual bee, K-means cluster analysis was used to group individuals as distinct morphs ("full-melanic" for bees with only black colouration or "banded" for bees with black, yellow and white colouration). I also calculated the percentage of body area that was black for each individual, to assess the variation in the degree of melanism.

Based on the data for body colour, the second aim of this chapter is to test a series of hypotheses related to those potential selective mechanisms outlined above that could be influencing colour morph frequencies, namely:

H1. The frequency of full-melanics and/or higher percent melanism scores increases as the ambient temperature and the number of sunshine hours at sites decreases. This hypothesis is based on the expectations related to thermal melanism.

H2. The frequency of full-melanics and/or higher percent melanism scores increases as the environment becomes more arid. This hypothesis is based on the result expected if melanism influences desiccation resistance.

H3. The frequency of full-melanics and/or higher percent melanism scores increases as the relative abundance of *B. hortorum* decreases. This hypothesis is based on the expected result if Müllerian mimicry is occurring.

To address the possibility that any difference in body colour between sites is the result of genetic drift, I compared the differentiation between populations observed at neutral microsatellite markers (Chapter 3) to levels of phenotypic differentiation, that is, the bee colouration measured here. Lastly, to consider the possibility that body colour in *B. ruderatus* is plastic, colour scores for individuals collected from Lake Tekapo in January and March were compared and the relationship between genetic relatedness and body colour was evaluated.

Methods

Sample collection

Samples were collected from Queenstown in January and Lake Tekapo in January and March in 2012 and samples from Nelson (Brightwater), Wanaka (Glendhu Bay), Alexandra, Waitaki River and Twizel were collected in January 2013. At each site *B. ruderatus* workers were collected as they were foraging and a tentative identification was made by close visual inspection. Collection of *B. ruderatus* continued until 30 to 35 individuals were collected. Leg tissue was taken from each individual and stored in 99% ethanol for DNA extraction. For more details about sampling locations and method refer to Chapter 2. As *B. ruderatus* is cryptic in colouration with *B. hortorum* and *B. subterraneus*, species identification was confirmed using a PCR based method (see Chapter 3 Methods: *Species identification*). Individuals were genotyped at 8 microsatellite loci to determine genetic structuring of populations and levels of relatedness, for more details refer to Chapter 3.

Photography

Each individual bee was photographed twice; both images were taken of the dorsum, one image of the thorax and the other of the abdomen. A consistent photography method was used as advised by Stevens *et al.* (2007). The corrections for bias associated with the camera were not used, therefore it should be noted that although measurements taken from the images allow for comparison of individuals within this study, they should not be used for comparison across studies (unless the exact same equipment and set up is used). All photographs were taken using a single Zeiss AxioCam HRc microscope camera, mounted on a Leica microscope with the following settings. The aperture in the microscope was set to open and the microscope was set at the coaxial position that blocked the light path to the eyepieces so that all of the light was directed to the camera. All of the photographs were taken under standardised lighting conditions. A lighting platform was constructed that fit into the base of the microscope, this housed four LED lights that were used to evenly illuminate the subject for each image (Figure 4.1). A voltage regulator was used to keep the lighting consistent at 9 volts and the lights were switched on and allowed to warm up for 30 minutes before images were taken. AxioVision v 4.3 was used to operate the camera. The exposure time was adjusted until no overexposure was detected at 70 ms and kept constant. The white balance was set using an Ocean Optics WS-1 diffuse reflectance standard and kept constant. All of the photographs were taken with the frame size at 1300 x 1030 pixels and the magnification set at 1.0x. Samples were cleaned of dust using an air pump and individuals that had wet and clumped

pile were not used. Files were saved in the Zeiss ZVI format and then converted into the 8-bit TIF format without image compression to avoid loss of data.

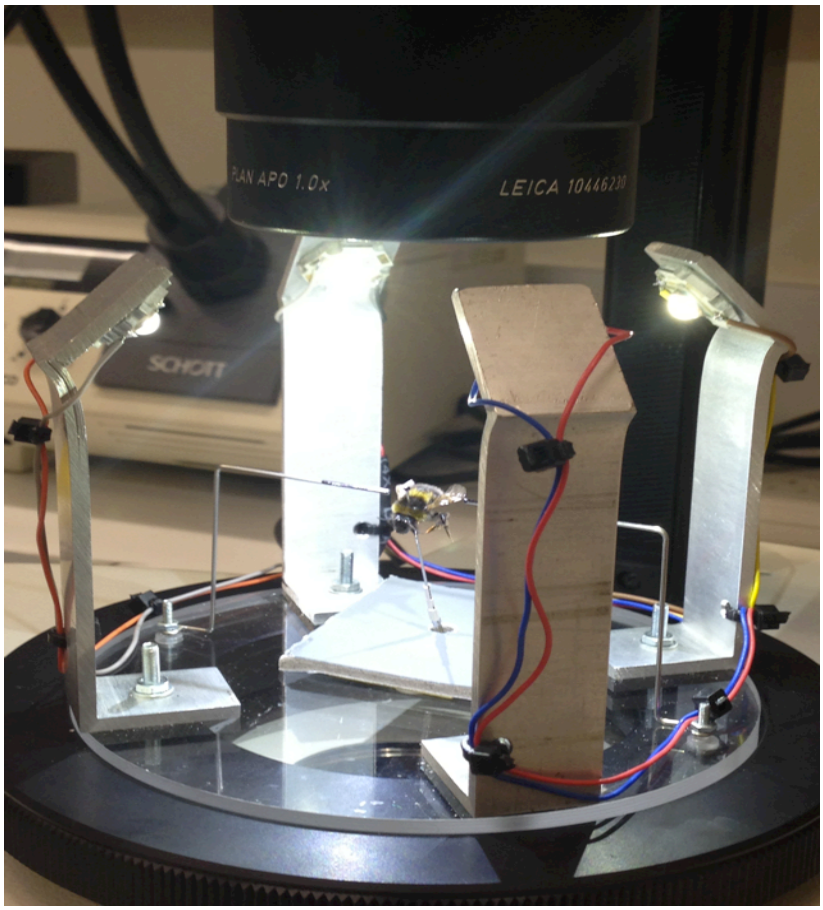

Figure 4.1: Microscope lighting set up used to take photographs for colour measurement. Four LED lights were used to evenly illuminate each worker bee.

Colour scoring

Colour data were measured from the TIF images for 183 *B. ruderatus* individuals and 20 *B. hortorum* individuals using the software ImageJ (Schneider *et al.* 2012). A point sampling grid was used for the thorax and the abdomen (Figure 4.2), measuring the red, green and blue (RGB) values for a total of 571 pixels on each individual. These raw RGB values were then converted into Colour 1 (R – G / R + G), Colour 2 (G – B / G + B) and luminance (R + G + B), as these values yield much more consistent results for subsequent analyses than using raw RGB or human vision-based colour spaces such as the Hue, Saturation and Lightness conversion (Endler 2012).

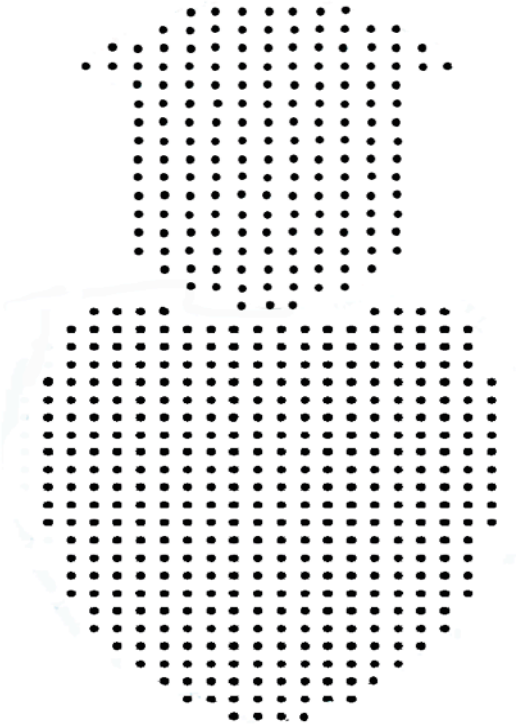

Figure 4.2: Sampling grid covering the bumble bee thorax and abdomen. A pixel was sampled at each point to measure Red, Green and Blue values.

The following analyses were conducted using R (R Development Core Team 2008). A K-means cluster analysis in combination with a principal component analysis (PCA) was used to identify grouping in the data. As the number of full melanic versus banded individuals within each population was of interest K (the number of final clusters) was set to 2. The PCA allowed the variation within clusters to be assessed and the results of the cluster analysis to be visualised. PCA biplots were created in R using the packages "ggplot2" and "ggbiplot". Histograms were used to look for the ranges of values associated with black, yellow and white areas. This allowed the classification of each point, and a "percent melanic" score for each individual to be calculated as the percentage of points in the grid with values characteristic of black areas, which the histograms showed to be luminance values < 115.

Impact of climate and conspecifics on body colour

To determine how climatic factors and mimicry may be affecting body colour, I utilised a model comparison approach in R, using the Second-Order Akaike Information Criterion (AICc) method (Burnham & Anderson 2002; Burnham & Anderson 2004) to compare models with the package "AICcmodavg". AICc is a form of AIC that is corrected for bias associated with small sample sizes

(Burnham & Anderson 2002). Two approaches were taken, the first set of models were binomial/logistic regressions, using binary data for colour grouping based upon the k-means cluster analysis with banded bees scored as 0 and full melanics scored as 1. The second set of models consisted of regressions using the percent melanic score outlined above for each individual as the measure of colour. The data had to be transformed by using the log of the percent melanic score so that the assumptions of normality and homoscedasticity were met.

Climate data used were from NIWA's CliFlo database, with values for each measure averaged over the months October through to March (see Chapter 2). To test the thermal melanism hypothesis, average daily maximum and minimum temperatures and monthly sunshine hours were used as predictors. To include sunshine hours in the model set, a second model set was run separately with the data from Twizel removed, as sunshine data were unavailable for this site. To test the hypothesis that melanism influences a bee's resistance to desiccation, average monthly relative humidity and average monthly total rainfall were used as predictor variables. To test the mimicry hypothesis, the relative abundance of *B. hortorum* (see Chapter 2) was used as a predictor variable. Models with cool-period, wet days, longitude and latitude as predictors were not included because of significant correlation and redundancy with some of the other predictors (Appendix 1). Finally both the binary and percent melanism model sets included a global model (with all the above predictors included) and a null model of no relationship (*i.e.* containing no predictor variables) for comparison (Table 4.1). To assess goodness of fit for each model, adjusted R^2 values or McFadden's pseudo R^2 values were calculated.

Table 4.1: Model set used to test hypotheses of adaptive function for melanism in *B. ruderatus*. Y = the response variable, which was either the proportion of 'full-melanics' (binary data) or percent melanism scores. The predictor 'sunshine hours' was included in a separate model set with the data for Twizel excluded because sunshine data were unavailable for this site.

Hypotheses	Model
Thermoregulation	Y ~ daily minimum temperature + daily maximum temperature (+ sunshine hours)
Desiccation tolerance	Y ~ rainfall + relative humidity
Müllerian mimicry	Y ~ proportion of *B. hortorum*
Global model	Y ~ daily minimum temperature + daily maximum temperature (+ sunshine hours) + rainfall + relative humidity + proportion of *B. hortorum*
Null model	Y ~ 1

Relative impacts of genetic drift and selection on body colour

To assess the relative role of genetic drift in influencing the divergence of body colour between *B. ruderatus* populations, F_{ST} as a measure of genetic differentiation was compared to P_{ST}, which is a measure of phenotypic differentiation. Genetic differentiation at quantitative trait loci (QTL) can be expressed as Q_{ST}, which is analogous to F_{ST} for selectively neutral loci, and the expectation is that $Q_{ST} = F_{ST}$ for neutral additive traits (McKay & Latta 2002). When QTL data are unavailable, an estimation of Q_{ST} can be calculated from direct measurements of the phenotype (P_{ST}) (Leinonen *et al.* 2006; Raeymaekers *et al.* 2007). Since F_{ST} is a measure of differentiation at selectively neutral loci, it provides a null where drift and gene flow are the only forces influencing P_{ST} (Leinonen *et al.* 2008). When $P_{ST} > F_{ST}$ we can conclude that phenotypic differentiation exceeds that which could be achieved by neutral processes alone, when $P_{ST} \approx F_{ST}$ then neutral processes cannot be ruled out, and when $P_{ST} < F_{ST}$ differentiation is less than that predicted by drift alone and this suggests that the phenotype is influenced by stabilizing selection (Merilä & Crnoknak 2001; Leinonen *et al.* 2008).

P_{ST} was calculated as in Leinonen *et al.* (2006):

$$P_{\mathrm{ST}} = \frac{\sigma_{\mathrm{GB}^2}}{\sigma_{\mathrm{GB}^2} + 2(h^2\sigma_{\mathrm{GW}^2})},$$

where σ_{GB}^2 is the variance between populations, σ_{GW}^2 the variance within populations and h^2 the heritability. I used the percent melanic score for each individual as the measure of phenotype. As phenotypic variation results from the influence of both genetics and the environment, it is unlikely that variation in phenotype is solely the outcome of additive genetic variation. Additive variation is due to the additive effect of genes, as opposed to non-additive variation that is due to interactions between genes such as dominance (Freeman & Herron 2007). Therefore, P_{ST} was calculated with two different h^2 values, with $h^2 = 1$ (all of the variation in phenotype is genetic and additive) and $h^2 = 0.5$ (half of the phenotypic variation is due additive genetic effects, and half is due to non-additive genetic and environmental effects). The value of $h^2 = 0.5$ was used because studies that have determined the heritability of melanism in insects have found values close to this (Nokelainen *et al.* 2013; Roff & Fairbairn 2013). Pairwise P_{ST} was also calculated and a Mantel test (Legendre & Legendre 1998) was implemented in GENODIVE v 2.0b23 (Meirmans & van Tienderen 2004) to look for a correlation between pairwise P_{ST} and pairwise F_{ST}.

Impact of phenotypic plasticity and the genetic basis of body colour

The usual method used to evaluate the influence of phenotypic plasticity is to conduct rearing experiments using genetically similar individuals across a range of environmental conditions in order to calculate a reaction norm (Freeman & Herron 2007). Alternatively, individuals from different phenotypic groups can be reared under the same conditions in common garden experiments (Freeman & Herron 2007). I attempted to rear bees by placing domiciles at two sites in Twizel but this was unsuccessful (see Appendix 2).

Due to the unsuccessful rearing experiments, samples collected from Lake Tekapo in January and March in 2012 were compared in order to assess phenotypic plasticity on body colour in *B. ruderatus*. The site at Mona Vale in Christchurch was also sampled in both months but no *B. ruderatus* were found. This approach allows a comparison of individuals from the same population that would have developed at a different time in the season and likely under different conditions. For instance, Lake Tekapo has a relatively large seasonal change in temperature over this period with an average reduction of 2.8 °C for both daily minimum and maximum temperatures from January to March. As there was no change in variation in selectively neutral genetic markers between these months ($F_{ST} < 0.001$ $P = 0.7$; Chapter 3), any change in colour morph frequencies is unlikely to be the result of selection, genetic drift or migration.

To look for an indication that melanism has a genetic basis in *B. ruderatus,* correlations were assessed between genetic relatedness and difference in percent melanism scores at both the population and individual level. Measuring heritability directly was outside the scope of this study, as this would require rearing colonies to provide access to individuals with known parent/offspring relationships. A Mantel test was implemented in GENODIVE to look for a relationship between genetic differentiation (pairwise F_{ST}: see Chapter 3) and melanism (pairwise difference in average percent melanic score) at the population level. Another Mantel test was used to look for a relationship between pairwise relatedness (Queller & Goodnight 1989) calculated in GenAlEx v6 (Peakall & Smouse 2006), and pairwise difference in percent melanic scores at the individual level for the 183 workers collected in January. The variation in colour scores of workers assigned to the same colonies (Chapter 3) is also assessed. If body colour has a clear genetic basis then little variation would be expected between sisters, as haplodiploid siblings are closely related to one another (relatedness 0.75) and monoandry is expected for *Bombus* species in New Zealand (Estoup *et al.* 1995). No relationship between genetic relatedness and melanism would suggest that this trait has low heritability which supports the hypothesis that body colour is plastic.

Results

Impact of climate and conspecifics on body colour

The results from the PCA of the colour of individual bees show two apparently distinct groups (Figure 4.3). One group consists of the "full-melanic" individuals and the other group consists of "banded" individuals as categorised by the K means cluster analysis. The first principal component explains 13.2% of the variation and characterises the degree of melanism across individuals, being strongly correlated with the percent melanic score (correlation coefficient = 0.95). The second principal component explains 4.0% of the variation and appears to characterise a difference in the yellow colouration across banded individuals (Figure 4.4). The PCA including the *B. hortorum* individuals shows that this species groups within the cluster of banded *B. ruderatus* (Figure 4.5). The proportion of full-melanic to banded individuals was significantly different across sites ($\chi^2 = 23.8$, critical value = 12.59, D.F. = 6, $P < 0.01$), with the greatest frequencies of melanic morphs at 0.29 occurring at Queenstown and 0.26 at Wanaka followed by Nelson at 0.14, compared to the remaining sites with frequencies between 0.07 and 0 (Figure 4.6). Differences in the percent melanic scores between sites were also significant (Figure 4.7A: ANOVA: $F_{6, 176} = 8$, $P < 0.001$). In order to assess whether an increase in the variation in percent melanic scores occurs at sites with a greater proportion of full-melanics in the banded bees, percent melanic scores for only bees from the banded group were plotted by site, confirming this trend (Figure 4.7B).

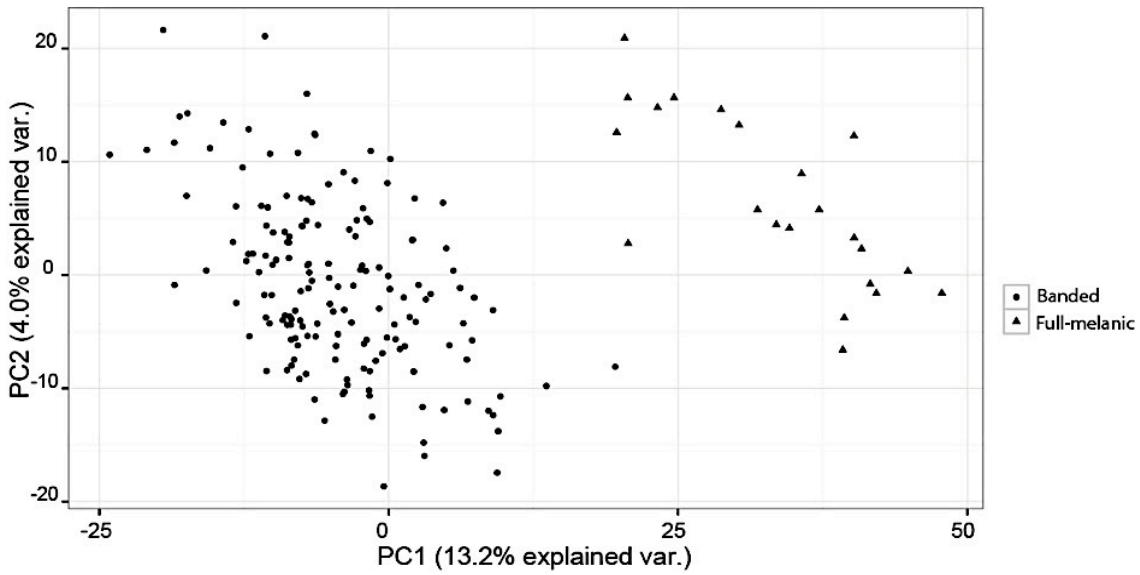

Figure 4.3: PCA biplot of the colour data for *B. ruderatus*. The results of the K means cluster analysis have been used to categorise "banded" individuals, making up the larger group on the left, and "full-melanic" individuals making up those individuals on the right.

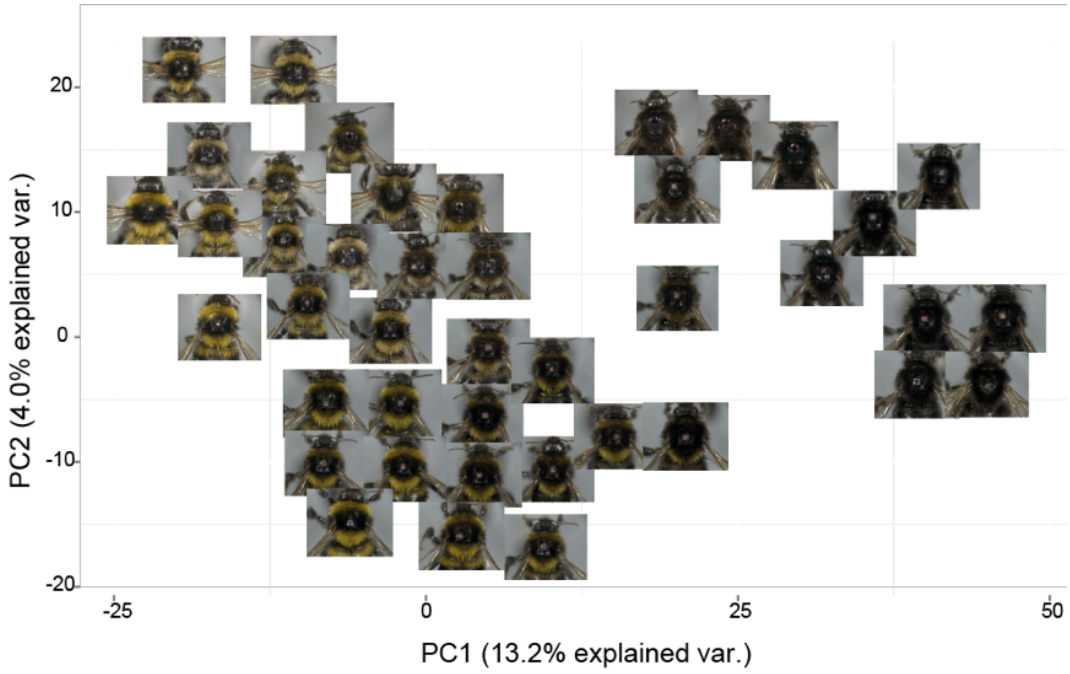

Figure 4.4: PCA biplot of the colour data for *B. ruderatus* with photographs showing the variation in body colour. PC1 describes the variation in melanism and was highly correlated to individual percent melanism scores. PC2 describes variation in yellow band colouration.

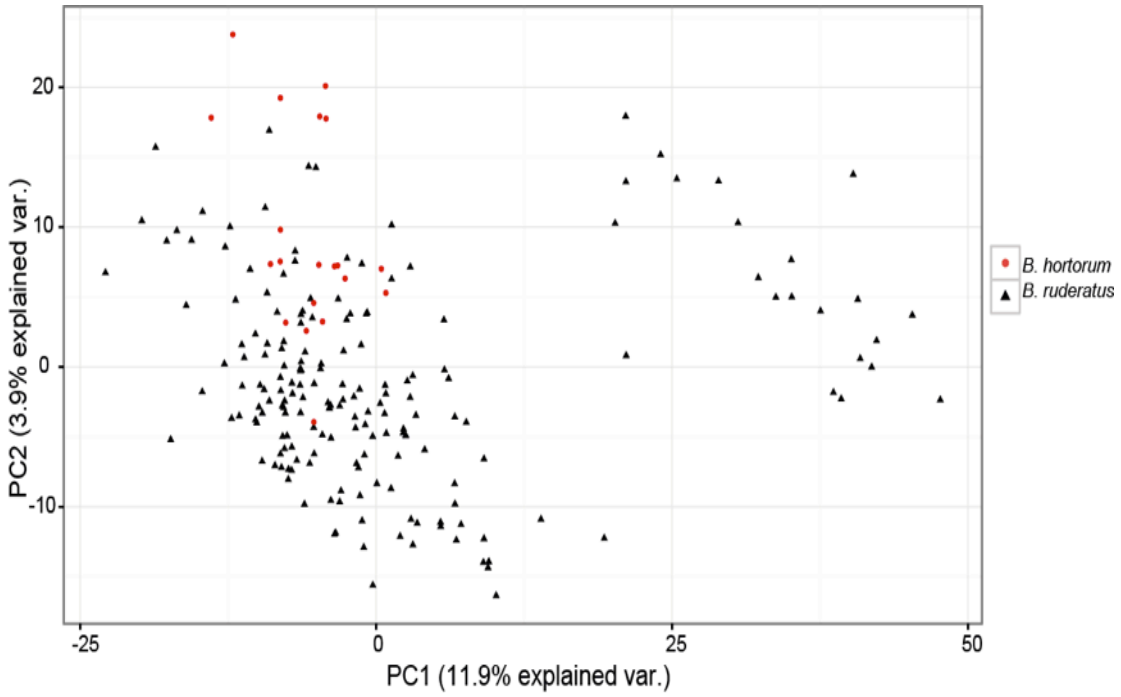

Figure 4.5: PCA biplot of colour data with *B. hortorum* individuals included. All of the *B. hortorum* individuals group within the banded *B. ruderatus* cluster on the left.

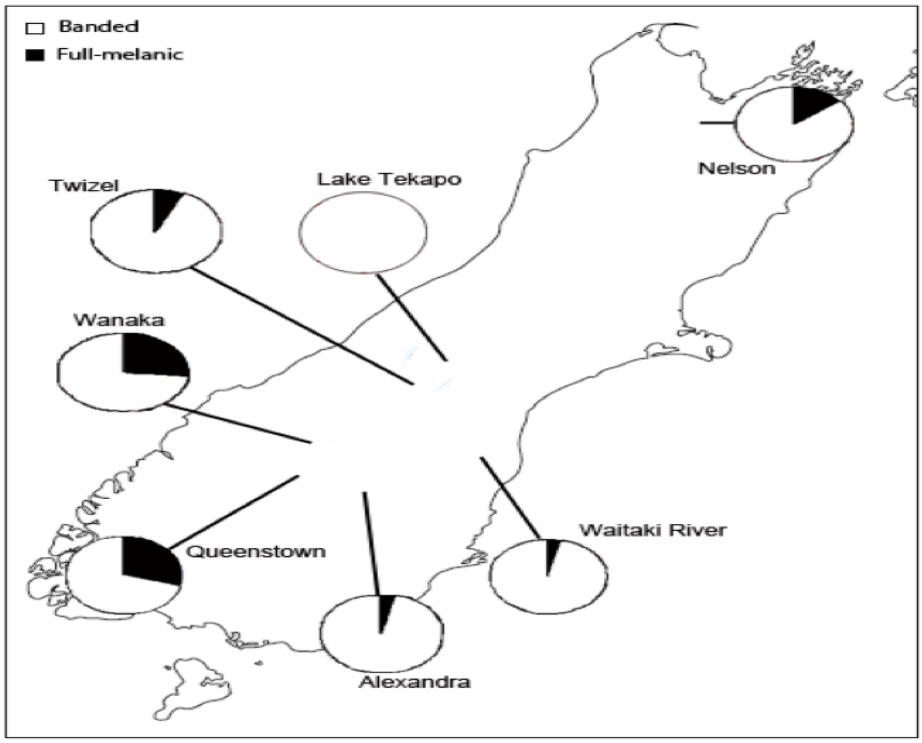

Figure 4.6: The proportion of full-melanic to banded *B. ruderatus* at sample sites in the South Island. The highest proportions of full-melanics were found at Queenstown (0.29) and Wanaka (0.26).

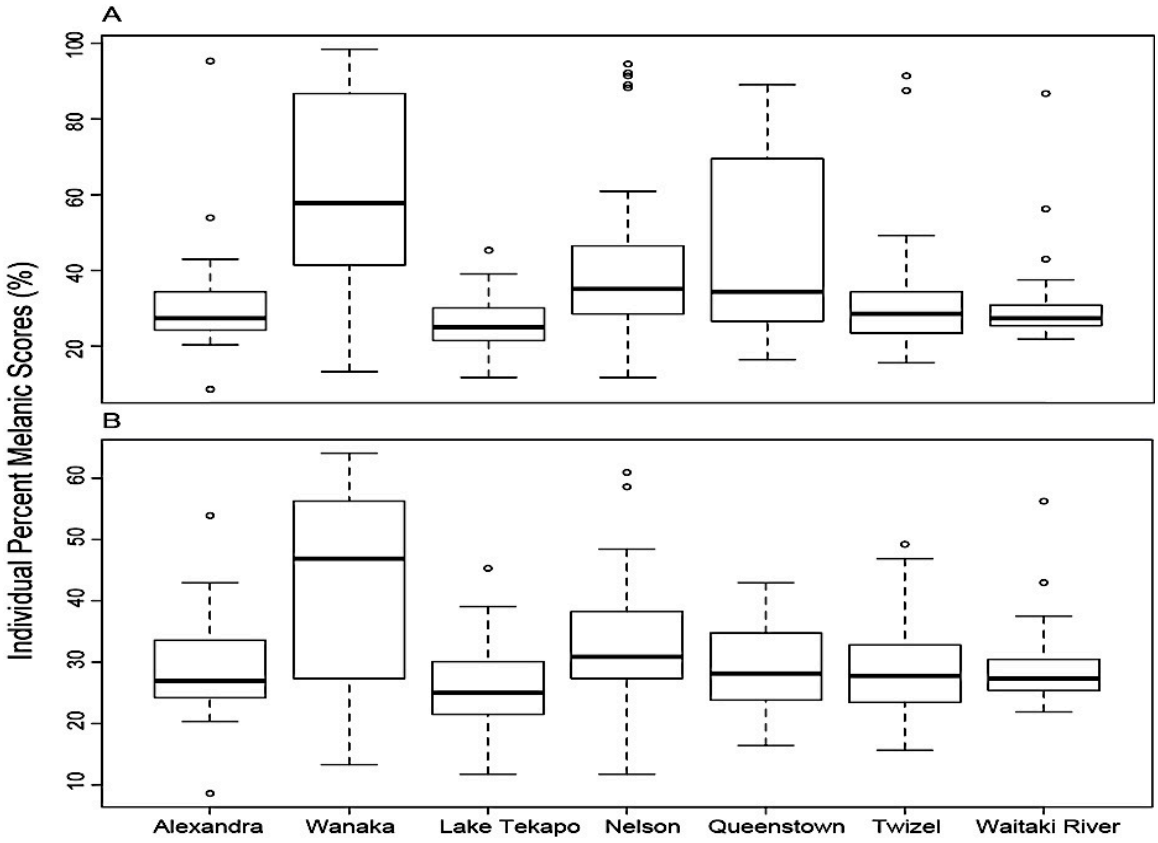

Figure 4.7: The range of melanism in A) all individuals and B) only individuals from the banded cluster, as shown by boxplots of individual percent melanic scores for each site. The heavy bar is the mean, with the box representing the 25th and 75th percentiles. The lines extend to values within 1.5 * the inter-quartile range, points are values outside this range and may be outliers.

The results for the AICc model comparison for the models using the proportion of full-melanic individuals as the response variable had the greatest support for the global model (Table 4.2). This was followed by the model with the proportion of *B. hortorum* as the predictor with a ΔAICc of 4.16 and humidity + rainfall with a ΔAICc of 6.25. The remaining two models had ΔAICc values greater than 10, indicating that there is essentially no support for these models and they do not add any extra information as shown by the Cumulative Weight column (Burnham & Anderson 2002; Burnham & Anderson 2004). The goodness of fit for these models was poor, with the best McFadden's pseudo R^2 value for the global model at 0.156, suggesting the global model explained approximately 16% of the observed variation in full-melanic bumble bees.

For the models that used the log of percent melanism for each individual as the response variable, the global model is the only supported model with a weight of 1.00 and the every other model has a ΔAICc value > 10 (Table 4.3). These models had similar goodness of fit, with the best adjusted R^2 value for the global model of only 0.154. When sunshine was included as a predictor variable in the model set, there was poor support for the temperature model with sunshine hours included as a predictor (similar to the temperature models in the previous analyses), although the weight of the humidity + rainfall model increased slightly for the set with the proportion of full-melanics as the response and now had the same amount of support as the global model with a ΔAICc of 0.22 (Appendix 3: Table A3.1). As the thermoregulation models with sunshine did not improve their ranking in the AICc tables and these models were run with data from Twizel excluded, the results from this are not discussed further.

Table 4.2: AICc table for the binomial regression models using the proportion of full-melanics (binary format) as the response variable. The global model includes all other predictors.

Model Predictor Variable(s)	K	AICc	Δ AICc	AICc Weight	Cumulative Weight	Log Likelihood	McFadden's pseudo R^2
Global Model	6	129.28	0.00	0.86	0.86	-58.40	0.156
Proportion of *B. hortorum*	2	133.53	4.16	0.11	0.97	-64.73	0.065
Humidity + Rainfall	3	135.53	6.25	0.04	1.00	-64.70	0.065
Null Model	1	140.41	11.13	0.00	1.00	-69.19	NA
Minimum + Maximum Temperatures	3	142.12	12.84	0.00	1.00	-67.99	0.017

Table 4.3: AICc table for the regression models using the individual percentage melanic score as the response variable. The global model includes all other predictors.

Model Predictor Variable(s)	K	AICc	Δ AICc	AICc Weight	Cumulative Weight	Log Likelihood	Adjusted R^2
Global Model	6	237.41	0.00	1.00	1.00	-111.39	0.154
Proportion of *B. hortorum*	2	254.22	16.81	0.00	1.00	-124.04	0.049
Humidity + Rainfall	3	257.04	19.62	0.00	1.00	-124.41	0.041
Minimum + Maximum Temperatures	3	257.95	20.53	0.00	1.00	-124.86	0.036
Null Model	1	262.48	25.07	0.00	1.00	-129.21	NA

These models give little direct support to the three specific hypotheses relating to different selective mechanisms that may be acting on body colour. The thermal melanism hypothesis predicted an increase in melanism with a decrease in ambient temperature and sunshine hours. The model with temperature (and sunshine) was consistently the predictive model with the least support for both model sets. Not surprisingly, there was little evidence of any effect of either minimum or maximum temperature on the global model, as indicated by both these variables overlapping zero in a plot of the parameter estimates (Figure 4.8). The desiccation resistance hypothesis predicted an increase in melanism in more arid environments. Again there was no strong relationship with either rainfall or humidity and there is little evidence that either of these variables had any effect on the global models, as these parameters also overlapped zero (Figure 4.8). The Müllerian mimicry hypothesis predicted that the frequency of melanic morphs would decrease as the proportion of *B. hortorum* increases. Although the model with the proportion of *B. hortorum* as a predictor was the only model to have any weight compared to the global model (Table 4.2), the general trend is the opposite to what was predicted with a significant positive effect size in the global model indicating more full-melanics where the relative abundance of *B. hortorum* was greater (Figure 4.8).

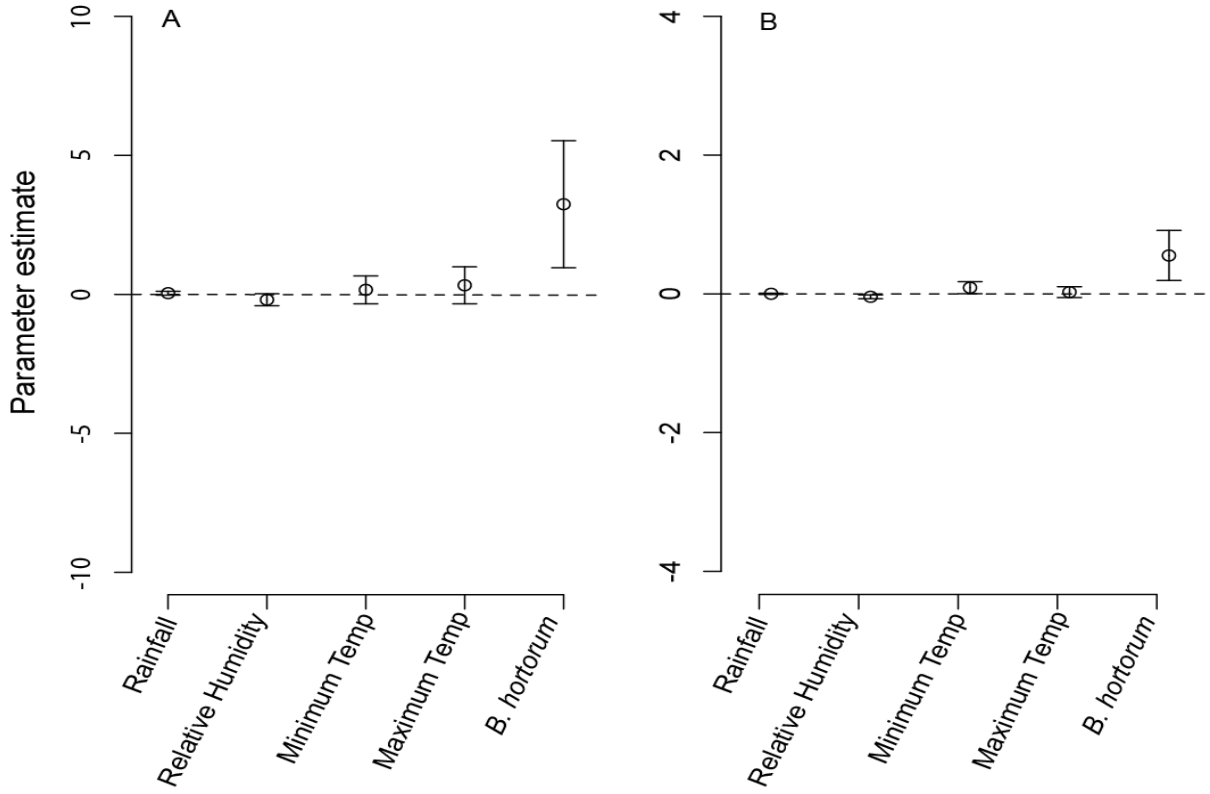

Figure 4.8: Effect sizes for each predictor from the global models with the response as either A) the proportion of full melanics (binary data) or B) percent melanism scores. Values are standardised parameter estimates ± 2 standard errors. Factors with error bars not overlapping with the zero line are considered significant effects ($P < 0.05$).

Relative impacts of genetic drift and selection on body colour

The overall P_{ST} value was 0.077 when $h^2 = 1$ and 0.144 when $h^2 = 0.5$. Both values are greater than the overall F_{ST} result of 0.035 (Results: Chapter 3). Pairwise P_{ST} values ranged from < 0.001 to 0.266 when $h^2 = 1$ and from < 0.001 to 0.420 when $h^2 = 0.5$. This differentiation appears to be driven by the large pairwise P_{ST} values for Wanaka compared to the other populations, as Wanaka has comparatively low to intermediate pairwise F_{ST} values (Figure 4.9). The Mantel tests for pairwise F_{ST} and pairwise P_{ST} had low Mantel's r-values and were non-significant for both $h^2 = 1$ (Mantel's r = -0.244, P = 0.264) and $h^2 = 0.5$ (Mantel's r = -0.241, P = 0.242).

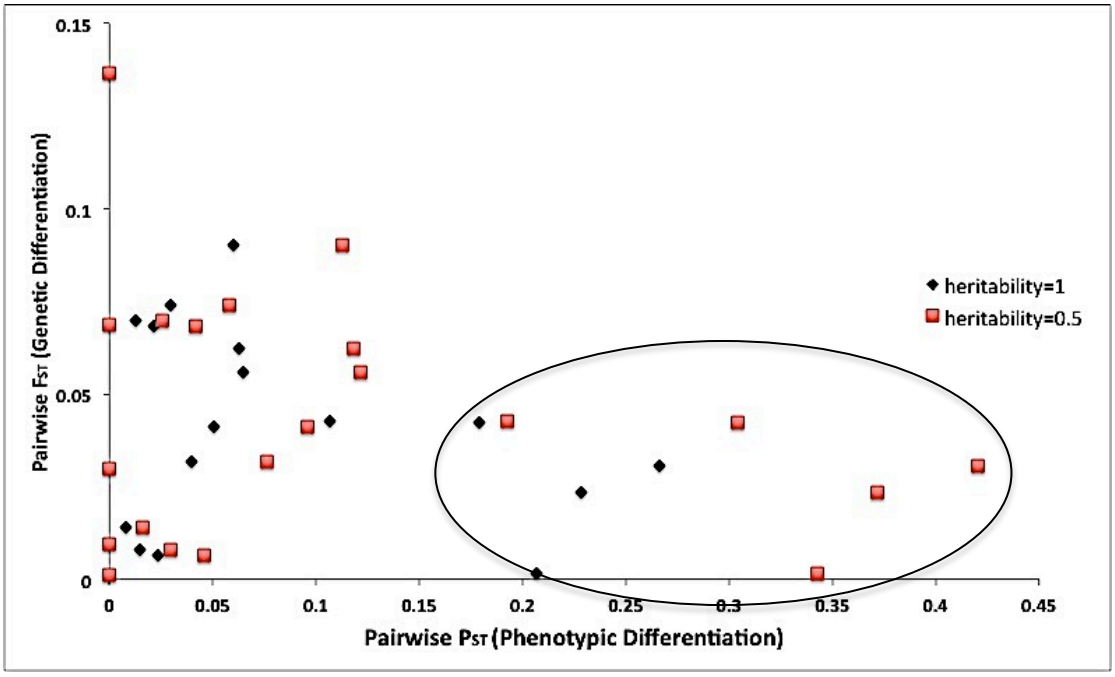

Figure 4.9: Scatterplot of pairwise F_{ST} versus pairwise P_{ST} for both levels of heritability. Mantel tests found no significant correlation for either heritability level. Points in the circle are pairwise comparisons with Wanaka.

Impact of phenotypic plasticity and the genetic basis of body colour

When the samples from Lake Tekapo that were collected in March were added to the PCA, two of the individuals were full-melanics and grouped with the 'full-melanic" cluster and the remaining bees grouped together as part of the banded cluster. However, the individuals collected in March appeared above the other banded individuals, as they generally had greater PC2 scores (Figure 4.10). The percentage of variance explained by the first principal component did not change at 13.2%, however there was an increase in the variance explained by the second principal component from 4 to 6.3%. There was a significant difference in the principle component scores between the

banded bees collected at Lake Tekapo in January and in March (PC1, ANOVA: $F_{1, 49} = 18.93$, $P < 0.001$; PC2, ANOVA: $F_{1, 49} = 102.46$, $P < 0.001$). As only two full-melanic bees were collected in March no analysis was carried out.

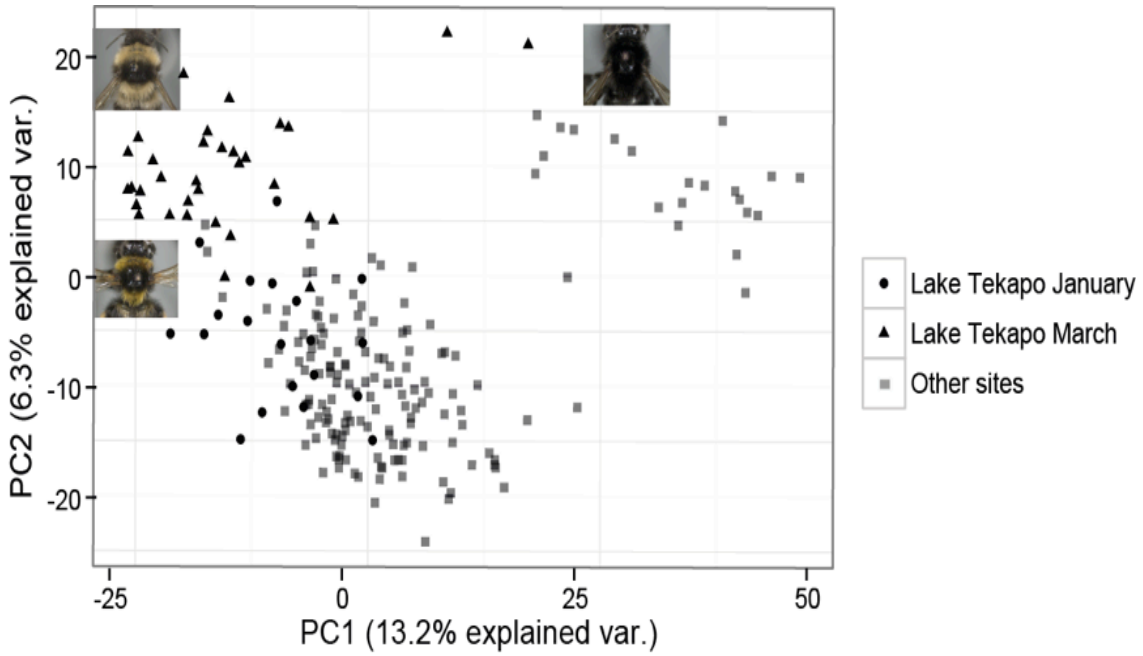

Figure 4.10: PCA biplot of colour data for *B. ruderatus* including the individuals collected from Lake Tekapo in March. There is an increase in the variation explained by PC2 (from 4% to 6.3%), indicating a difference in the yellow band colouration between January and March. Two of the workers collected in March were also full-melanics.

The Mantel test between pairwise F_{ST} and pairwise difference in the average percent melanic score at the population level was non-significant (Mantel's r = -0.077, P = 0.533; Figure 4.11). The Mantel test between pairwise relatedness and pairwise difference in percent melanism at the individual level had a significant Mantel's r-value of -0.06 (P = 0.008). This fact that this result is significant is likely due to the large sample size involved in the test as the r-value indicates that the correlation is extremely weak. The majority of colonies were represented by a single individual; therefore this result may also reflect a lack of variation in the relatedness data to test for a relationship with melanism. Of the 54 colonies that were represented by more than one worker, 27.8% of these colonies contained both full-melanic and banded workers. The majority of these colonies were represented by only two workers, however in one case seven workers were grouped together as part of the same colony from the Nelson samples. The individuals from this colony ranged in percent melanism from 19% through to 89% with an average of 36%.

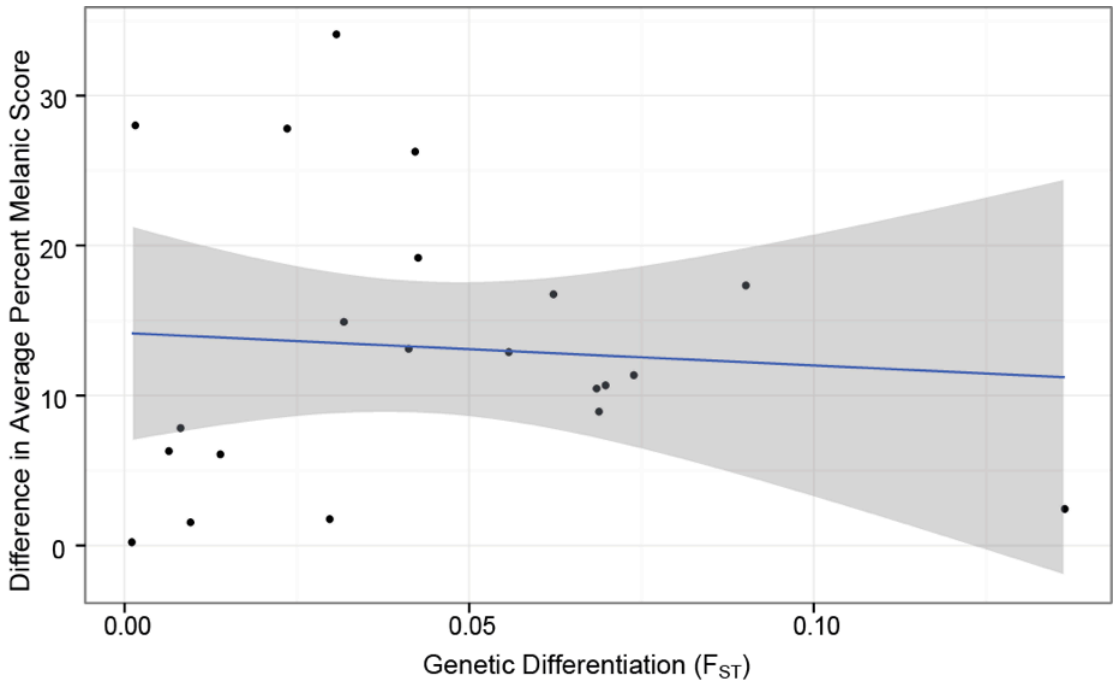

Figure 4.11: Mantel correlation between pairwise genetic differentiation (F_{ST}) and pairwise difference in the average percent melanic score. Mantel's r = -0.077, P = 0.533. The shaded area is the standard error.

Discussion

Previous studies of bumble bee colouration have relied on observer-based scoring of templates, a method that makes it difficult to quantify continuous variation. Using digital photography in this study allowed the objective scoring of colour at many points in a sampling grid, thus allowing a continuous measurement of melanism. Lozier *et al.* (2013) used a similar photography method and found continuous colour variation within the distinct colour morphologies of *B. bifarius* in the USA. The PCA supports the presence of distinct colour morphs, with individuals grouping into two clusters, although there is continuous variation in the degree of melanism and in the yellow colouration itself (Figure 4.4). While there is no broad geographical pattern over the entire South Island, the variation in melanism does not appear to be randomly distributed over the sites in the central South Island (Nelson excluded), as the Lake Tekapo, Waitaki River, Twizel and Alexandra sites have a proportion from 0 to 0.07 full-melanic individuals whereas the proportion of full-melanics at Wanaka and Queenstown is approaching 0.3 (Figure 4.6). Sites with a greater proportion of full-melanics also have more banded individuals that show a greater degree of melanism (Figure 4.7). This suggests that an increase in full-melanic morphs is complemented by an increase in melanism of banded morphs.

The cause of this pattern in melanism remains unclear, as the data does not support any of the specific hypotheses regarding the selection for or against melanism. If melanism was important for thermoregulation in *B. ruderatus*, an increase in melanism is expected with colder ambient temperatures and lower sunshine. This is was not the case, with no trend for maximum temperature or sunshine hours and if anything there was a weak positive relationship with minimum temperature. Melanism may not be as important for thermoregulation in bumble bees as in other insects because of other thermoregulatory adaptations. Bumble bees are able to regulate their body temperature by physiological means through shivering thoracic flight muscles and dissipating heat through their abdomen to prevent overheating (Heinrich 1974; Heinrich 1979). It is also possible that no pattern was found because the sites sampled do not represent low enough ambient temperatures for melanism to become important for thermoregulation in bumble bees. Pekkarinen (1979) noted that melanic forms were mostly common in species with a mainly alpine range in Scandinavia. As the long tongued species in New Zealand do not forage on native species (See Chapter 2), *Bombus ruderatus* is likely to be somewhat restricted from alpine environments because of the prevalence of native vegetation at higher altitudes (Mark *et al.* 2000).

There was also no support for the desiccation resistance hypothesis. If melanism was important for increasing desiccation resistance then melanism should be more prevalent in arid environments. There was no relationship between relative humidity and melanism and a weak trend of increased melanism with greater average total monthly rainfall. Desiccation resistance based on cuticular permeability is probably more important for smaller bodied insects such as *Drosophila* than large bodied insects (Le Lagadec *et al.* 1998). The lack of correlation for all of the climate variables could also be because the weather data were regional based values from weather stations. The effect of climate at the macroclimatic scale may be less important than the influence of microclimate. Therefore it is possible that a relationship with regards to thermoregulation and desiccation resistance was not found because the climate data measured were too coarse.

The third specific hypothesis was that colour pattern in *B. ruderatus* would be associated with Müllerian mimicry. The only model that had any weight other than the global models in the AICc analyses was the model with the proportion of *B. hortorum* as the predictor when the proportion of full-melanics was the response (Table 4.2). However, this model had less support than the global model with a ΔAICc > 4 (Burnham & Anderson 2002; Burnham & Anderson 2004). In addition to this the trend was in the opposite direction to that predicted; with an increase in melanism as the relative abundance of *B. hortorum* increases (Figure 4.8). Figure 4.5 illustrates that *B. hortorum* has

colouration that matches the banded *B. ruderatus;* therefore Müllerian mimicry would suggest a positive relationship between the two banded morphs and not the observed positive relationship of *B. hortorum* with full-melanic *B. ruderatus*. I suspect that this may be a confounded result that is due to correlation between the relative abundance of *B. hortorum* and another factor that is actually influencing melanism in *B. ruderatus.* For instance, the distribution of *B. hortorum* does appear to be influenced by climate (Chapter 2) and while there is little evidence of climate affecting melanism in *B. ruderatus*, it is an example of the complexity of interactions of the factors that may be driving colour in *B*. *ruderatus*.

Given that aposematism seems to have been important in the evolution of colour patterns in bumble bees, why would Müllerian mimicry not be operating between *B. ruderatus* and *B. hortorum* in New Zealand? Stelzer *et al.* (2010) transplanted colonies of different *B. terrestris* subspecies from populations in the UK, Germany and Sardinia. These subspecies show variation in body colour, from yellow and black bands with a white tail, to mostly black with a white tail or mostly black with a red tail. They found that native populations did not consistently have lower loss rates of workers than transplanted colonies (in fact the opposite was recorded in Sardinia), suggesting that unfamiliar colouration did not expose transplanted bees to greater predation risk (Stelzer *et al.* 2010). These results suggest that, although mimicry may have an important influence on bumble bee colouration in some cases, it may not be the only (or most important) factor influencing convergence of body colour in bumble bees (Stelzer *et al.* 2010). Selection imposed by visual predators on bumble bees in New Zealand could be relaxed in comparison to their native range. Colour polymorphism in the aposematic jersey moth is only found where it was introduced to England, and is thought to be the result of a non-visual mechanism acting on these populations where selection imposed by predators has become relaxed (Brakefield & Liebert 1985). Goulson (2003) suggests that the success of bumble bees in New Zealand is likely to be partly due to the loss of natural enemies. Selection on colour pattern signals may also be relaxed if the general shape and auditory signals (that would be common across bumble bees) are distinctive enough that predators learn to avoid bumble bees even when their colour patterns vary. The “warning buzz” used by bumble bees was shown to be an effective aposematic signal when bees attempted to take over nest boxes occupied by birds (Jablonski *et al.* 2013). So perhaps selection for warning colouration in bumble bees introduced to New Zealand has been reduced.

The results from the F_{ST} to P_{ST} comparison showed that overall phenotypic differentiation between sites was greater than neutral genetic differentiation, with the overall P_{ST} values ($h^2 = 1$, $P_{ST} = 0.077$;

h^2 = 0.5, P_{ST} = 0.144) exceeding the F_{ST} of 0.035. There was no significant correlation between pairwise P_{ST} and pairwise F_{ST} values. When the pairwise values were looked at in detail the only comparisons where $P_{ST} \approx F_{ST}$ occurred when both values were low and when h^2 = 1. Several of the comparisons had a $P_{ST} > F_{ST}$ (particularly when h^2 = 0.5), however it is apparent that the overall increase of P_{ST} is driven by the large differentiation between Wanaka and the remaining populations except for Queenstown. On the other hand, several comparisons that had low pairwise P_{ST} values (between 0 and 0.05) showed greater levels of neutral genetic differentiation (Figure 4.9). These results could suggest that divergent selection between populations where $P_{ST} > F_{ST}$ or stabilising selection between populations where $P_{ST} < F_{ST}$ is the likely explanation, although further interpretation of this should be made with caution (Pujol *et al.* 2008). As P_{ST} is estimated from phenotypic data the relative variance due to environmental and non-additive genetics components is unknown, therefore the observed pattern could be the result of phenotypic plasticity (Pujol *et al.* 2008). Alho *et al.* (2010) found that the pattern of melanism in common frogs follows a latitudinal cline in Sweden and Finland aligning with the expectations of the thermal melanism hypothesis and they found that P_{ST} was greater than F_{ST}. However, common garden experiments found that melanism was largely the result of phenotypic plasticity; therefore the observed pattern of divergent selection was actually the result of plasticity (Alho *et al.* 2010).

What evidence is there that phenotypic plasticity is influencing body colour in *B. ruderatus*? The PCA scores for both the first and second principal components are significantly different for workers collected in January and March at Lake Tekapo (Figure 4.10). There is a prominent change in the band/yellow colouration as indicated by the increase in variation described by principal component two when the March samples are included. This seasonal change in colouration could be the result of phenotypic plasticity. There is no change in the microsatellite allele frequencies between January and March (Chapter 3), which suggests that a change in colour morph frequencies is unlikely to be due to migration of individuals into the population. In fact since it is the queens that disperse and new colonies are not founded until the following year a change observed in workers cannot be due to incoming migrants. Selection for different colouration could cause a shift in colour morph frequencies over this time, caused by the differential success and failure of some colonies with different coloured workers over the season. However, this seems unlikely as this would require strong selection for the lighter colour to cause the large change observed and no evidence for strong selection based on the factors discussed in this chapter was found.

It is possible that the observed overall lightening of colour has been caused by sun bleaching or 'fading' of colour with age (Hooper *et al.* 1999; Kemp 2006). This would require that the workers collected in March were older and had experienced more exposure to sunshine than those collected in January. It is difficult to estimate the age of workers collected in the field. Many of the workers collected in March had considerable wing damage, however this has been shown as an unreliable estimator of age in bumble bee workers (Foster & Cartar 2011). The longevity of adult workers varies between species from approximately 2 to 6 weeks, although most workers are only expected to live for a week or two due to high mortality associated with foraging (Goulson 2003). Workers start out only performing tasks within the nest (such as nursing larvae) and are more likely to become foragers as they get older (Goulson 2003). Worker longevity was found to decrease as colony age increased in *B. bifarius,* which is probably because the age those workers first began foraging decreased as colonies became older (O'Donnell *et al.* 2000). If foraging mortality is associated with predation then bumble bee workers may have greater life expectancy in New Zealand than in their native range. In New Zealand, nest founding for *B. ruderatus* occurs over a four-month period from October to January (Donovan & Weir 1978). Whilst a seasonal change in age structure is possible, you might expect that more long-lived sun bleached individuals from nests founded earlier in the season would have been collected from any of the sites in January.

There was no relationship between relatedness and difference in percent melanism at the individual level (Mantel's r = -0.06) or between pairwise F_{ST} and pairwise difference in average percent melanism scores (Mantel's r = -0.077). These results suggest that melanism in *B. ruderatus* has a low heritability. This analysis did not include the workers collected in March and so the result is not due to the seasonal change observed at Lake Tekapo. If melanism were under strong genetic control then more closely related individuals would be expected to have more similar levels of melanism. It would be interesting to assess levels of variation in body colour within colonies; unfortunately I was unable to collect data from whole colonies because no colonies were initiated in the nest boxes that were put out at the Twizel site (see Appendix 2). However, some insight can be gained from the foraging workers collected as colony membership can be assigned by recreating sibships from the microsatellite data (Chapter 3). This confirmed that some colonies contain workers that show a range of melanism. Variation in colour between closely related siblings from the same haplodiploid parents (relatedness = 0.75) suggests that this trait has low heritability, supporting the hypothesis that rather than having a genetic basis, variation in melanism is more likely the outcome of phenotypic plasticity.

In summary, whilst a significant pattern of melanism was discovered across populations of *B. ruderatus* in the South Island, it is unclear what may be driving this pattern. The comparison of phenotypic and selectively neutral genetic differentiation suggests that differences between populations are greater than the differentiation that could be achieved through genetic drift alone. This suggests that either selection or phenotypic plasticity is influencing melanism in *B. ruderatus*, or perhaps both. The AICc modelling approach did not reveal any model that fit the data well and suggested that a global model with every predictor as the best. This could mean that there is a general pattern between climate and colour pattern and the driver of this pattern was not included as a predictor in the models. What the data does outline is that thermoregulation, desiccation resistance and Müllerian mimicry do not appear to influence melanism in *B. ruderatus* in the South Island. While there is reason to believe that pile colouration is under genetic control in bumble bees, the range of melanism observed in *B. ruderatus* suggests that the underlying genetic basis of melanism may be complex and involve multiple loci. A seasonal change in body colour was also detected at Lake Tekapo, although it is unclear if this is due to phenotypic plasticity or is related to sun bleaching in older individuals. Closely related worker bees from the same colonies can have different levels of melanism, which also suggests some environmental influence on colour development. Taken together, these results imply that patterns of melanism across *B. ruderatus* populations are complex and it is likely that multiple factors are influencing melanism in concert.

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Chapter 5: General discussion

Melanism in Bombus ruderatus

Variation in melanism in *B. ruderatus* was continuous within the banded bees through to a distinct group of individuals that were full-melanics. The degree of melanism differed significantly between sites in the South Island. The proportion of full-melanics was greatest at Wanaka and Queenstown followed by Nelson ranging from 0.29 to 0.14, with melanism almost absent at the remaining sites where frequencies of full-melanics ranged from 0.07 to 0. Differences in percent melanic scores (the percentage of points on each individual that were melanic) followed the same pattern across sites (Chapter 4).

The pattern in the frequency of melanic morphs at different sites does not appear to be solely the result of random changes. The evidence for this comes from the comparison of phenotypic differentiation (P_{ST}) to genetic differentiation at selectively neutral loci (F_{ST}) (Chapter 4). This shows that overall phenotypic differentiation is greater than the differentiation at microsatellite loci. The pairwise comparisons showed that $P_{ST} > F_{ST}$ in some cases and $P_{ST} < F_{ST}$ in others. This suggests that the difference in melanic morph frequencies is greater between some sites and less between others than the difference expected if frequencies were only changing at random.

If drift is not the only mechanism influencing the frequency of melanism in *B. ruderatus,* as some sites are more or less different than expected by chance, then a reasonable conclusion is that selection might be having an effect. Globally, colour patterns of different species were found to group geographically (Williams 2007), which suggests that there are several adaptive functions for body colour in bumble bees. I tested three hypotheses for the adaptive function of melanism in *B. ruderatus* in New Zealand. The first hypothesis was that melanism would influence thermoregulation, the second hypothesis was that melanism would increase desiccation tolerance and the third hypothesis, based upon Müllerian mimicry, was that melanism would be maladaptive at sites where *B. hortorum* (a species with similar colouration to the non-melanic forms of *B. ruderatus*) was relatively abundant.

Melanism is expected to influence thermoregulation in insects because dark or black colouration increases the rate of heating in colder ambient temperatures compared to lighter coloured individuals at a given level of solar radiation (True 2003; Clusella-Trullas *et al.* 2007). Two authors have previously investigated 'thermal melanism' in bumble bees. Pekkarinen (1979) looked at

variation in a number of morphological features as well as allozymes in several bumble bee species in Scandinavia. This author found that the frequency of melanic morphs was positively correlated with the number of 'cool' days in spring for *B. hortorum* in Sweden, suggesting that queens would benefit from thermal melanism in these colder temperatures when foraging during the early stages of nest development. Stiles (1979) was interested in the trade-off between thermoregulation and mimicry in sexually dimorphic bumble bee species. He found that sexual dimorphism in the length, density and colour of the pile was more prevalent in colder latitudes north of 30°N. Males at these latitudes had longer pile that should aid in retaining warmth whilst resting on flowers at night, but less dense pile and lighter colouration that should help to prevent overheating during sustained flight in the day.

The results from the AICc modelling in Chapter 4 gave no support for the model that used only the measures of temperature (minimum temperature was significantly correlated with cool period, see Appendix 1) and sunshine as predictors of melanism. There was also no increase in the frequency of melanic morphs at colder temperatures, which would be expected under the 'thermal melanism' hypothesis. These results suggest that melanism does not have an important affect on thermoregulation in *B. ruderatus* at the sites sampled in the South Island. Bumble bees have a number of adaptations that influence how they regulate body temperature that are reviewed by Heinrich (1979). Whilst bumble bee pile is known to function as insulation (Heinrich 1979), how pile colour influences thermoregulation has not been determined experimentally. Bumble bees are also able to generate heat internally through the shivering of flight muscles and as such can be considered endothermic insects (Heinrich 1974; Heinrich 1979). This means that bumble bees are not reliant upon basking in the sun in order to heat up, which could explain why melanism does not appear to be adaptive for thermoregulation in *B. ruderatus* in New Zealand. It is also possible that no pattern was found because the sites sampled do not represent low enough ambient temperatures for melanism to be important for thermoregulation in bumble bees. Pekkarinen (1979) noted that melanic forms were mostly common in species with a mainly alpine range in Scandinavia. Bumble bees in New Zealand are likely to be somewhat restricted from alpine environments because of the prevalence of native vegetation at higher altitudes (Mark *et al.* 2000).

Melanism has been found to affect desiccation tolerance in *Drosophila* (Brisson *et al.* 2005; Parkash *et al.* 2008; Rajpurohit *et al.* 2008; Ramniwas *et al.* 2013). This is because melanism influences the permeability of insect cuticle resulting in increased water retention (Ramniwas *et al.* 2013). As such, darker coloured individuals are expected to have greater fitness in more arid environments.

Bombus ruderatus is more abundant in areas with drier and warmer climates in New Zealand, which is similar to Europe where it has a more southerly distribution than *B. hortorum* (Macfarlane & Gurr 1995; Lye *et al.* 2010). Lye *et al.* (2010) also found that *B. ruderatus* foraged mainly in the middle of the day, when conditions are at their hottest and driest in New Zealand. Melanism could increase the desiccation resistance of workers foraging in these conditions. However, the results from the AICc models in Chapter 4 gave little support for the model using rainfall and humidity as predictors. Melanic morphs were actually more prevalent in Queenstown and Nelson, the sites with characteristically high rainfall relative to the other sites. Water retention may be more important for smaller bodied insects than for relatively large bodied bumble bees (Le Lagadec *et al.* 1998). In addition, pile colouration may not actually have as much influence on how permeable the cuticle is as the pigmentation of the underlying cuticle (which is black bumble bees). Lastly, bees in general are thought to combat desiccation through high metabolic water production during flight, which is substantial for larger species such as bumble bees (Nicolson 2009).

The third hypothesis for the adaptive function of body colour was that Müllerian mimicry between *B. ruderatus* and *B. hortorum* would cause the frequency of melanism to decrease where *B. hortorum* was relatively abundant. Müllerian mimicry is thought to be important in the evolution of bumble bee colour patterns (Plowright & Owen 1980; Williams 2007; Hines & Williams 2012). A pattern of strongly contrasting bands of yellow, red and black colour, considered aposematic because it increases conspicuousness, is the most common colouration in bumble bee species (Williams 2007). The results from the AICc analysis in Chapter 4 suggested that the only model that had any support apart from the global model was the model that used the relative abundance of *B. hortorum* as a predictor. However, the trend is the opposite of that predicted, with an increase in melanism where *B. hortorum* is more abundant. The relative abundance of *B. hortorum* was greater at Wanaka and Queenstown, where melanism was more prevalent, than at Twizel and Lake Tekapo where a low frequency of melanics was recorded. Lye *et al.* (2010) found a similar pattern of relative abundance for these two bumble bee species in this area, suggesting that sampling effort was adequate to draw the above conclusion. The relative abundance of *B. ruderatus* and *B. hortorum* is linked to climate in the South Island (Chapter 2). Therefore, the positive correlation between melanic morphs in *B. ruderatus* and the abundance of *B. hortorum* may be a confounded result, due to the abundance of *B. hortorum* being correlated with another factor that is actually influencing melanism in *B. ruderatus*.

Why then would Müllerian mimicry not be operating between *B. ruderatus* and *B. hortorum* in New Zealand? One answer is that the predators that exert selection for aposematic colouration on bumble bees in their native ranges may not be present in New Zealand. One of the reasons that bumble bees have been successful in New Zealand is likely the lack of natural enemies following introduction (Goulson 2003). There aren't any specialised avian predators of bumble bees in New Zealand (Donovan & Weir 1978), and birds are the predators typically thought to exert selection for aposematic colouration in bees because they are visual predators (Chittka & Osorio 2007). However, there are several parasites including an internal mite, a nematode, a protozoan and two external mite species that attack *Bombus* which were accidentally introduced to New Zealand with bumble bees (Donovan 1980). The movement of parasites between countries in commercially exported bumble bee hives is a significant threat to both managed and native pollinator populations (Graystock *et al.* 2013). Melanism has actually been linked to parasite and disease resistance in insects (Bailey 2011; Dubovskiy *et al.* 2013). The physiological pathway for the production of phenoloxidases (one of the compounds involved in immune response in insects) is the same for melanin production (Wittkopp & Beldale 2009). Another common immune response in insects is encapsulation of a foreign entity by surrounding it with a melanised 'crust' (Bailey 2011). Encapsulation and phenoloxidase activity have both been reported in bumble bees (Moret & Schmid-Hempel 2000; Moret & Schmid-Hempel 2001). Whether melanic forms of *B. ruderatus* or any *Bombus* species are more resistant to parasites or diseases is unknown. However, parasites and diseases could be linked to the abundance of *B. hortorum*, which may explain the observed trend of an increase in melanism in *B. ruderatus* in the presence of greater numbers of *B. hortorum*.

One process that will weaken the observed evidence for any relationship between melanism and selection is gene flow, through reintroducing morphs that are selected against and therefore altering colour morph frequencies. The results from Chapter 3 were used to determine the genetic structure of *B. ruderatus* populations in the South Island. These results suggested that Nelson bumble bees are genetically isolated and receive little to no gene flow from the other sites. Out of the remaining sites, Queenstown grouped with Wanaka, and Twizel grouped with Lake Tekapo and Waitaki River, in terms of their genetic similarity. Alexandra was intermediate between these two groups. This genetic structuring has similarities with the pattern of melanism in the central South Island. The sites with the greatest proportion of fully melanic bees were Wanaka and Queenstown followed by Nelson, with the remaining sites having a relatively low proportion of melanics. The frequency of melanic bees at Alexandra is lower than expected based on the genetic structure, given that it was intermediate in the cluster analysis and not significantly differentiated from either

Wanaka or Waitaki River. One possibility here is that selection is influencing the effective migration and therefore gene flow into Alexandra from Wanaka. The most polymorphic site for the degree of melanism was Wanaka, which may be the result of a balance between selection for melanism at Wanaka and dispersal of non-melanic bees from Alexandra. No migrants or their direct decedents were detected in the STRUCTURE analysis, although the distances between these sites meant that detecting migrants was unlikely. The shortest distance between sites in this study was approximately 30 km, and although no estimates of dispersal distance have been calculated for *B. ruderatus*, the maximum suggested dispersal distance of *B. pascuorum* and *B. lapidarius* queens is likely to be about 10 km (Lepais *et al.* 2010).

Although there was no support for any of the specific hypotheses for the adaptive function of melanism in *B. ruderatus* in the South Island, it seems likely that selection is still influencing the frequencies of different morphs to some extent. The global model for both model sets (using either the proportion of totally melanic bees or percent melanism scores as the response variable) explained approximately 15% of the variation in melanism (Chapter 4). This seems relatively low, however in this case is still likely to be biologically meaningful. Given that there is a range of possible functions for body colour and it is likely that a combination of these influence fitness (Chapter 1), melanism in *B. ruderatus* is probably affected by a range of environmental factors that were measured and others that were not. It is also possible that the pattern of selection is masked by the effect of gene flow between some populations as well as the history of relatively recent introduction and range expansion (Chapter 3). In addition to this, melanism in *B. ruderatus* shows continuous variation within the banded morph type, and heritability is unlikely to be 100% for this trait (Chapter 4). Therefore, while no single predictor that was measured appears to be a strong driver, collectively these variables explain a reasonable proportion of the variation in melanism. While this relationship between the environment and melanism could be driven by either selection or plasticity, because the climate data used were long term averages this result is more likely to reflect adaptation over several generations as opposed to a plastic change during development in response to the environment within the generation that was sampled. This suggests that selection may be responsible for at least part of the observed pattern of divergence between populations.

Whilst divergent selection could explain the observed difference in the frequency of melanism between sites, another possibility is that some of the variation in melanism is due to phenotypic plasticity. Plastic traits can be adaptive, with variation due to plasticity mirroring patterns that are expected under divergent selection (Alho *et al.* 2010). Although there is likely to be some genetic

basis for melanism in bumble bees (Owen & Plowright 1980; Owen *et al.* 2010), several results suggest that melanism has a low heritability in *B. ruderatus* (Chapter 4). There was no significant correlation between the difference in average percent melanism and genetic differentiation at the population level and a very weak correlation between genetic relatedness and difference in percent melanism across all individuals. In addition to this, in some cases individuals that were grouped together into full-sibling groups showed the full range of melanism. The results from the comparison of colour between individuals from the same site collected in January and March also suggested a seasonal change of pile colour occurs in *B. ruderatus* (Chapter 4). Rather than a change in the degree of melanism, this change appears to be primarily an increase in the overall lightness of pile colour, particularly in the yellow component. This result may be due to the effect of sun bleaching of the colour in older workers or could be due to a change in environmental conditions over the season, such as a change in pollen quality or availability.

One environmental factor that has been found to influence colour development in insects is temperature (Goulson 1994; Michie *et al.* 2010; Yamamoto *et al.* 2011; Green *et al.* 2012). However, temperatures that bumble bee larvae develop in are likely to be more regulated than for many non-social insects (Heinrich 1979; Goulson 2003), and thus plasticity in response to temperature during development is unlikely in bumble bees. The nest itself provides a thermoregulated environment, often constructed from the remains of abandoned rodent nests combined with wax secretions to form a layer of insulation that traps metabolic heat produced by the queen and workers (Heinrich 1979; Goulson 2003). The queen and workers also actively incubate the developing larvae (Heinrich 1979; Goulson 2003).

Although temperature is regulated in bumble bee nests, one factor that is more likely to vary between developing larvae is diet. While *B. ruderatus* in New Zealand forages almost exclusively on only two species (*Echium vulgare* and *Trifolium pratense*) (Goulson & Hanley 2004; Lye *et al.* 2010), changes to pollen availability or pollen quality (Delph *et al.* 1997) of these species over the season could potentially affect colour development. Condition dependent expression of body colour based on diet has been found in several insect species (Kemp 2008; Punzulan *et al.* 2008; Canfield *et al.* 2009; Tibbets 2010). Bumble bees species are divided into two groups with respect to how they feed developing larvae. The 'pocket makers' push pollen into a groove underneath the developing 'brood clump' that houses multiple larvae and then the pollen is grazed upon (Goulson 2003). By comparison in the 'pollen-storing' species, the brood clump is broken up into individual cells and each larvae is fed individually (Goulson 2003). In all bumble bee species there is considerable

variation in body size between sister workers from the same nest, with the growth of larvae directly proportional to the food they receive (Goulson 2003). A difference in the amount of food received by larvae based on their position in the nest is probably the cause of the variation in worker size for pollen-storing species (Couvillon & Dornhaus 2009; Couvillon *et al.* 2010). In pocket-makers (such as *B. ruderatus*), competition for food between developing larvae within the same brood clump likely results in worker size variation in these species (Goulson 2003). Diet clearly influences development of body size in adult bumble bees but how food intake relates to colour development is unknown. Synthesis of melanin pigment is expected to be costly for insects (Roff & Fairbairn 2013); therefore resources available to larvae during development could influence overall levels of melanin production. Pollen may also provide the pigment (or its precursor) for the yellow colouration in bumble bees (Hines 2008).

Bumble bee conservation

The decline of some bumble bee species in their native ranges has been documented in Europe (Goulson *et al.* 2008) & North America (Cameron *et al.* 2011). In the UK, *B. terrestris* and *B. hortorum* are still relatively common, whereas both *B. ruderatus* and *B. subterraneus* have declined in Europe, with *B. subterraneus* now extinct in the UK (Goulson *&* Hanley 2004). By studying bumble bees in New Zealand, some aspects of the biology of the declining species have been discovered that would have been difficult to observe at their current abundance in the UK. For instance, differences in the time of day that species forage (Lye *et al.* 2010), and the main forage species utilised by bumble bees in New Zealand (Goulson & Hanley 2004; Lye *et al.* 2010) have been documented. These findings can help inform management strategies for the conservation of bumble bees in the UK (Lye *et al.* 2010).

In New Zealand, Goulson & Hanley (2004) found that the abundance and distribution of *B. ruderatus* and *B. subterraneus* appears to have reduced since the survey by Macfarlane & Gurr (1995). The abundance of *B. subterraneus,* in its restricted range around lake margins in the South Island, appears to have continued to reduce from its numbers in 2003 through to 2008-2009 (Lye *et al.* 2010). Although in New Zealand bumble bees are introduced (indeed 'invasive') species, there are two reasons why there is an interest in the persistence of bumble bee populations. Firstly, bumble bees provide a pollination service for several commercial crops (Howlett & Donovan 2010). Secondly, populations of *B. ruderatus* and *B. subterraneus* in New Zealand could provide a source for re-introduction to their origin, which was planned for *B. subterraneus* (Lye *et al.* 2010).

The abundance and distribution of each of the *Bombus* species in the central South Island (Chapter 2), was similar to that found by previous research (Goulson & Hanley 2004; Lye *et al.* 2010). Results from the searches conducted in gardens suggest that only *B. terrestris* and *B. hortorum* utilise the floral resources at these sites in New Zealand. I also sampled several areas where bumble bees have not been investigated since Macfarlane & Gurr (1995). A reasonable abundance was recorded for each of the three species (*B. subterraneus* not present) recorded at the two Nelson sites and the Waitaki River site (Chapter 2). These same species were also present at Lake Coleridge where both main forage species (*Trifolium pratense* and *Echium vulgare*) were abundant; although *B. hortorum* was dominant here in both years the site was sampled (Chapter 2). These results show that *B. ruderatus* has persisted outside of the Mackenzie Basin and, in the case of the inland Nelson site, was the most abundant species present.

There was an absence of *B. ruderatus* in north Canterbury and the Christchurch area (except for a single worker collected at the Mona Vale garden in March) although this species was recorded in this area by Macfarlane & Gurr (1995). The cause of this apparent decline in Christchurch is unclear, as *B. hortorum* and *B. terrestris* are still present; perhaps changes to agricultural practice or an increase of urban areas in this region are responsible. Windy weather prevented searches along the eastern coastline near Kaikoura and Seddon, although the dry climate here should favour *B. ruderatus* and I think it would be interesting to conduct counts in this region. Why some species remain common while others have declined is one of the biggest debates and mysteries for bumble bee conservation, with some authors suggesting that forage availability is the key driver (Goulson *et al.* 2005; Goulson *et al.* 2008) and others suggesting that broader differences in climate and habitat preference is a better predictor (Williams 2005; Williams *et al.* 2009). There is a correlation between emergence time and decline, with species that have queens that emerge from hibernation to found colonies later in the season more likely to be in decline (Fitzpatrick *et al.* 2007; Williams *et al.* 2009). In New Zealand, *B. ruderatus* emerges later in the season than *B. hortorum* (Donovan & Weir 1978); as such competition with the established *B. hortorum* populations for resources or nesting sites may be important.

The results from Chapter 3 suggest that most of the populations sampled are stable, as levels of genetic diversity remain comparable to those in non-declining species in their native ranges. The recent range reduction in *B. ruderatus* probably reflects the loss of populations from some areas where changes to habitat quality have occurred, while the remaining populations are at locations such as Lake Tekapo and Wanaka, where good habitat for bumble bees has remained. The

population at Queenstown may be more vulnerable than the others due to its relative isolation and the detection of a significant inbreeding coefficient. Evaluating the prevalence of sterile diploid males in this area would give an indication of how this inbreeding is influencing fitness in the population. The results from the circuit theory modelling (Chapter 3) suggested that habitat plays an important role at a broad scale in facilitating dispersal between populations and this finding may help inform management strategies for some bumble bee species in their native ranges.

Recommendations

My findings suggest that melanism doesn't have a strong adaptive function with regards to thermoregulation, desiccation tolerance or aposematism, but do suggest that a combination of factors that likely includes factors that were not measured, is influencing the frequency of melanism in *B. ruderatus* in the South Island. There is also some evidence that suggests the environment influences colour development in this species. This presents more questions than it answers, which warrant further investigation.

Before any firm conclusions can be made about the influence of selection, the genetic basis of body colour in *B. ruderatus*, and bumble bees in general, should be clarified. Previous work focused on a discrete component in the colour pattern of one species, and suggests in this case either black or red colouration has a relatively simple genetic basis (Owen & Plowright 1980; Owen *et al.* 2010). Black or red/orange colour results from changes made during the same developmental pathway, with melanin responsible for both of these colours in bumble bees (Hines 2008). The pigment responsible for yellow colour in bumble bees is difficult to characterise, however it is common across species and is not a melanin (Hines 2008). In species like *B. ruderatus* that have a range of intermediate melanic/yellow forms, the underlying genetic basis for body colour may be more complex. Determining the molecular basis, both the genes responsible and how they are expressed would be the ultimate goal in this regard. For now, studies that calculate the heritability (h^2) of body colour in species with more complex colour patterns are needed. Experiments that use a common garden approach, or manipulate environmental conditions during development to evaluate plasticity would also be valuable.

While I was able to test specific hypotheses for the adaptive function of melanism in *B. ruderatus* there are other possible functions that were outside the scope of this study. The pattern of increasing melanism where the relative abundance of *B. hortorum* is greater is counter to expectations if mimicry based on predation by visual predators was important. This pattern could

be linked to a non-visual mechanism, either linked to climate or perhaps increased parasite or disease resistance. Inducing the immune response in *B. terrestris* workers that were under starvation conditions had a significant affect on survival compared to controls (Moret & Schmid-Hempel 2000), therefore parasite resistance is likely to influence fitness. How parasite loads vary between individuals, nests and populations with different levels of melanism, and how parasite abundance is linked to the relative abundance of different bumble bee species could be investigated.

Another possibility is that body colour is important for the survival and reproductive success of male or queen bumble bees. Factors influencing the survival of males and queens during the essentially solitary part of the life cycle when they are searching for mates and when queens are founding colonies at the beginning of the following season are likely to be different to those for workers. For instance, the immune response was found to differ between worker and male *B. terrestris* (Moret & Schmid-Hempel 2001), which suggests different selective pressure in regards to parasite resistance. In addition to survival, colouration could influence reproductive success, used as a signal to attract mates and be under the influence of sexual selection. Courtship and mating interactions are notoriously difficult to observe in bumble bees due to the difficulty in tracking individuals (Goulson 2003). Bumble bees use colour to help discriminate between flowers when foraging (Raine & Chittka 2007), and can discriminate between the same or different objects based upon colour suggesting they posses an identity concept (Brown & Sayde 2013). To address these possibilities future work on *B. ruderatus* should also assess the frequency of different colour morphs in reproductive individuals.

For future studies on colour polymorphism in *B. ruderatus* in the South Island, I would suggest that they were conducted over a smaller spatial scale. Sampling a transect from Wanaka (the most polymorphic site) over the ~30 km through to Alexandra (where melanic forms were rare) may reveal more about what is driving the increased frequency of melanism in Wanaka. At this scale, potentially important microclimatic factors could be measured and the measures suggested above should be included. A transect with sampling at intervals < 10 km apart allows the identification of migrants (were population genetics incorporated), which would provide further insight into the balance between divergence and gene flow.

Summary

Whilst a pattern of melanism was discovered across populations of *B. ruderatus* in the South Island, it is unclear what may be driving this pattern. The comparison of phenotypic and selectively neutral genetic differentiation suggests that differences in melanism between populations are greater than the differentiation that could have been achieved through genetic drift alone. This suggests that either selection or phenotypic plasticity is influencing melanism in *B. ruderatus*, or perhaps both. Thermoregulation, desiccation resistance and Müllerian mimicry do not appear to drive differences in the frequency of melanism in *B. ruderatus* in the South Island. However, while no single predictor that was measured appears to be a strong driver, collectively these variables explained a reasonable proportion of the variation, which suggests selection is operating to some extent. This could mean that there is a general pattern between climate and colour pattern and the driver of this pattern was not included as a predictor in the models, with resistance to parasites one possible adaptive function that was not tested. The results from Chapter 4 also suggest that the heritability of melanism may be low and that body colour in *B. ruderatus* is a plastic trait that is probably influenced by environmental conditions during development. Perhaps climate influences the availability and quality of pollen, which in turn influences the development of colour in *B. ruderatus*. Taken together, these results imply that patterns of melanism across *B. ruderatus* populations are complex and it is likely that multiple factors are influencing melanism in concert.

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Appendix 1

Two measures of climate were excluded from the analyses in Chapter 4 due to co-linearity between predictor variables caused by correlation between predictors (Table A1). The measure "cool period" was removed from analyses because it was correlated with both measures of daily temperature. The measure "wet days" was excluded because it was correlated with rainfall. As cool period and daily minimum and maximums were measures of temperature and wet days and rainfall were measures of precipitation the excluded variables were redundant. The measures Latitude and Longitude were also excluded from the models in Chapter 4 due to strong significant correlations with several of the climate measures. Although several of the remaining variables showed a relatively high correlation, these were not excluded as they represented distinct hypotheses.

Table A1: Correlation coefficients for measures of climate and geographic position. Correlations significant after Bonferroni correction are in bold.

Climate measures	Daily minimum	Daily maximum	Rainfall	Cool period	Wet days	Humidity	Latitude	Longitude
Minimum	0							
Maximum	0.097	0						
Rainfall	**0.687**	-0.007	0					
Cool period	**-0.750**	**-0.649**	**-0.635**	0				
Wet days	**0.491**	**-0.353**	**0.839**	**-0.302**	0			
Humidity	**0.525**	**-0.435**	**0.258**	-0.121	**0.529**	0		
Latitude	**0.735**	-0.132	**0.734**	**-0.519**	**0.435**	**0.280**	0	
Longitude	**0.688**	**-0.275**	**0.581**	**-0.414**	**0.408**	**0.537**	**0.923**	0

The number of wet days was defined as the number of days per month with rainfall > 1 mm. The average number of wet days per month between the years 1993 and 2012 was compared for the regions Alexandra, Wanaka, Queenstown, Nelson, Oamaru, Lake Tekapo, Twizel and Christchurch. The spring "cool period" was determined by looking at histograms of daily minimum and maximum temperatures. These followed normal distributions, therefore days that had a recorded minimum

and maximum temperature below the average were considered “cool days”. The cool period (defined as the number of days with a minimum temperature < 5 °C and a maximum temperature < 15°C) was compared for October and November between the years 2003 and 2012 at Alexandra, Lake Tekapo, Nelson, Oamaru, Queenstown, Wanaka and Twizel.

The average number of wet days differed significantly between regions ($F_{7, 130} = 7.41$, $P < 0.0001$). The lowest average numbers of wet days were observed at Lake Tekapo, Alexandra, Christchurch, Twizel and Wanaka ranging from 6.2 to 6.7. Oamaru, Nelson and Queenstown had on average the highest number of wet days at 7.3, 7.5 and 8.2 (Figure A1.1).

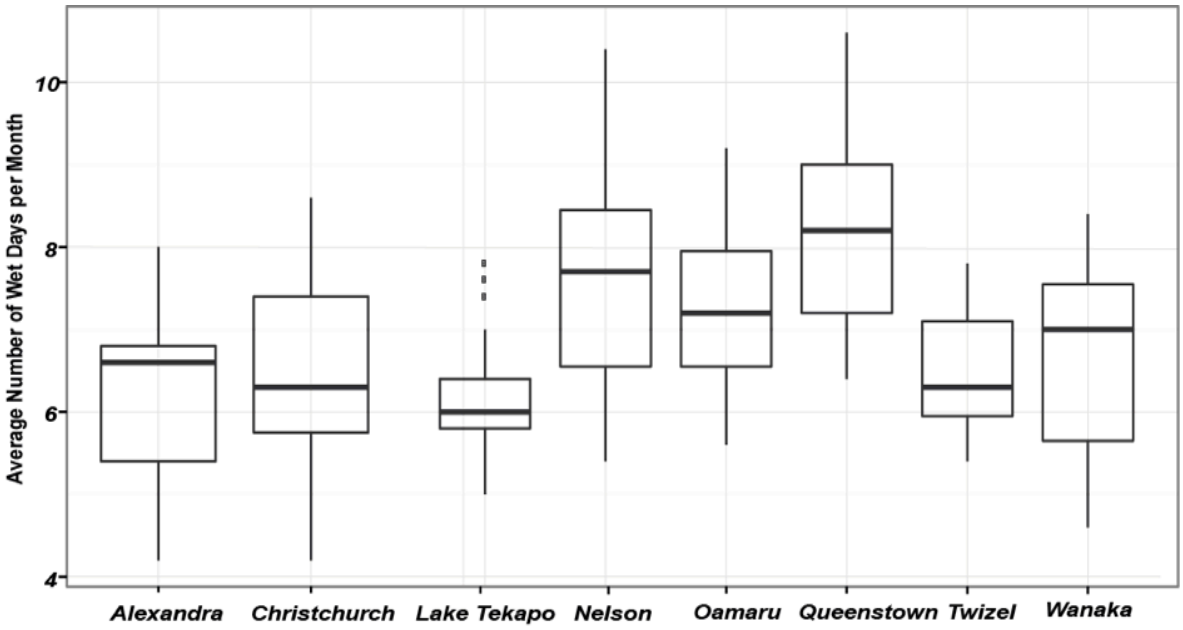

Figure A1.1: Differences in the average number of wet days per month between regions for the months October through to March. Data were averaged for the period from 1993 to 2012. The heavy bar is the mean, with the box representing the 25th and 75th percentiles. The lines extend to values within 1.5 * the inter-quartile range, points are values outside this range and may be outliers.

The average spring cool period differed significantly between sites in both October (logistic regression: $P<0.001$, D.F. = 6) and November (logistic regression: $P < 0.001$, D.F = 6). The number of cool days at all sites was lower in November than in October. In October, Lake Tekapo had the greatest number of cool days with an average of 15.6. Oamaru, Wanaka, Queenstown and Twizel had average cool periods from 10.9 to 8 days. The regions with the least number of cool days in October were Alexandra and Nelson with 4.9 & 2.1 days respectively. In November, Lake Tekapo

and Oamaru still had average cool periods of 6.5 and 5.5 days respectively, whereas the remaining regions had between 2.7 and 0.3 days (Figure A1.2).

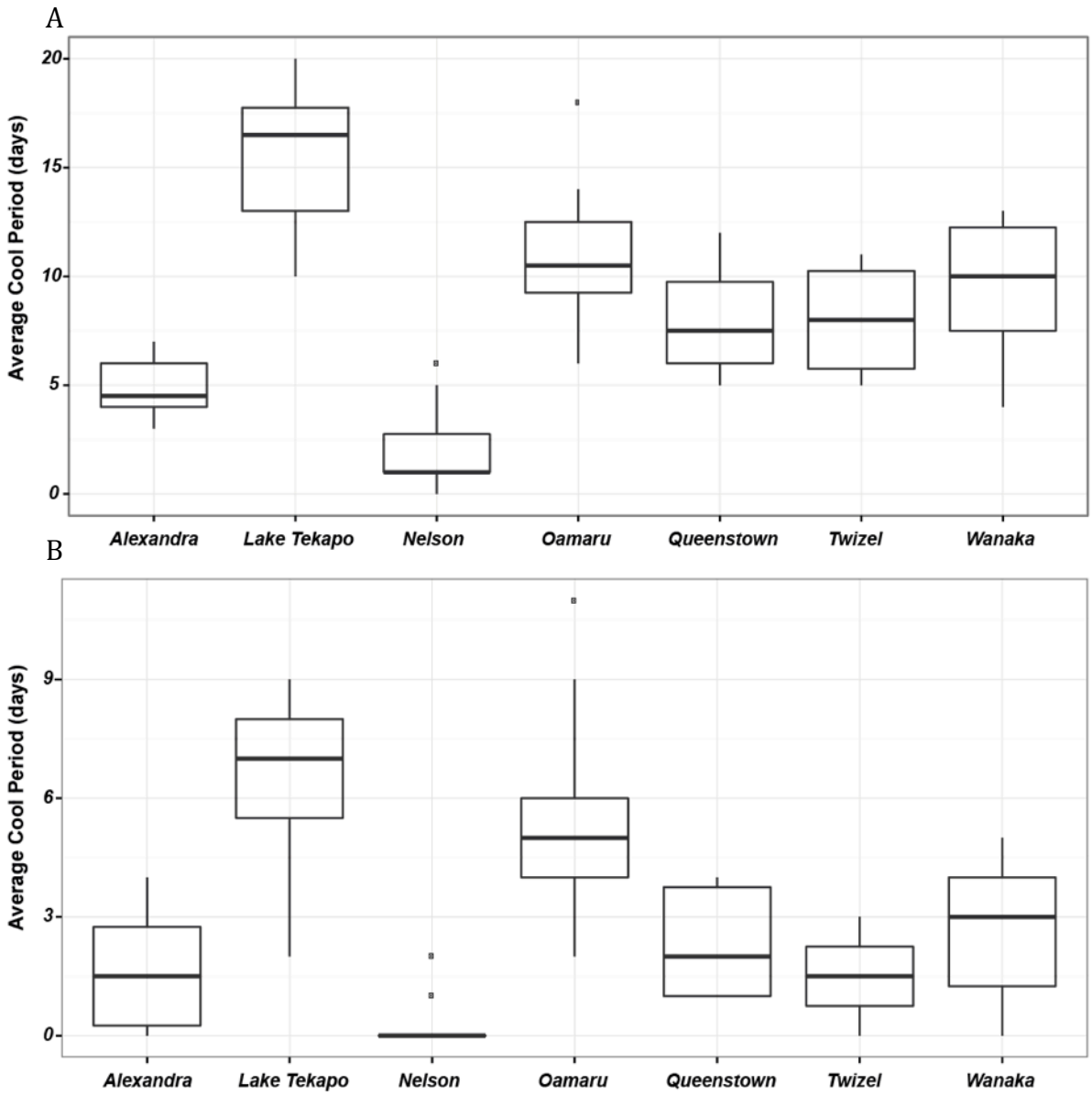

Figure A1.2: Differences in the average "cool period" (defined as the number of days with a minimum temperature < 5°C and maximum temperature < 15°C) between regions in A) October and B) November. Data were averaged for the period from 1993 to 2012. The heavy bar is the mean, with the box representing the 25th and 75th percentiles. The lines extend to values within 1.5 * the inter-quartile range, points are values outside this range and may be outliers.

Appendix 2

Nest boxes

Two sites near Twizel; The Wairepo Arm and Ruataniwha Wetlands were chosen for the placement of bee boxes based on recommendation from bumble bee researcher, Barry Donovan, who has had previous success with nests in this area. Nest boxes were constructed using a 9 L polystyrene box (Long Plastics Ltd) that was partially buried (to a depth approximately half the height of the box) and then covered with earth to block out light. A bamboo tube (~ 25 cm long, 2 cm internal diameter) leading from the surface down at an approximately 55° angle to a hole cut into the box was used to create a tunnel entrance. Small holes were punched into the base of the box to allow drainage of any moisture. Nesting material placed inside the box consisted of straw and cotton wool, shaped to recreate something similar to a rodent nest (Figure A2.1). A total of 32 nest boxes as well as a single large wooden domocile with four nesting compartments (each of similar dimensions to the polystyrene boxes) were placed in early November. Each nest box was checked for occupation by looking into the box with a fibre optic light scope inserted down the length of the bamboo tunnel in both December and again in late January. No occupation of nest boxes was recorded by any *Bombus* species.

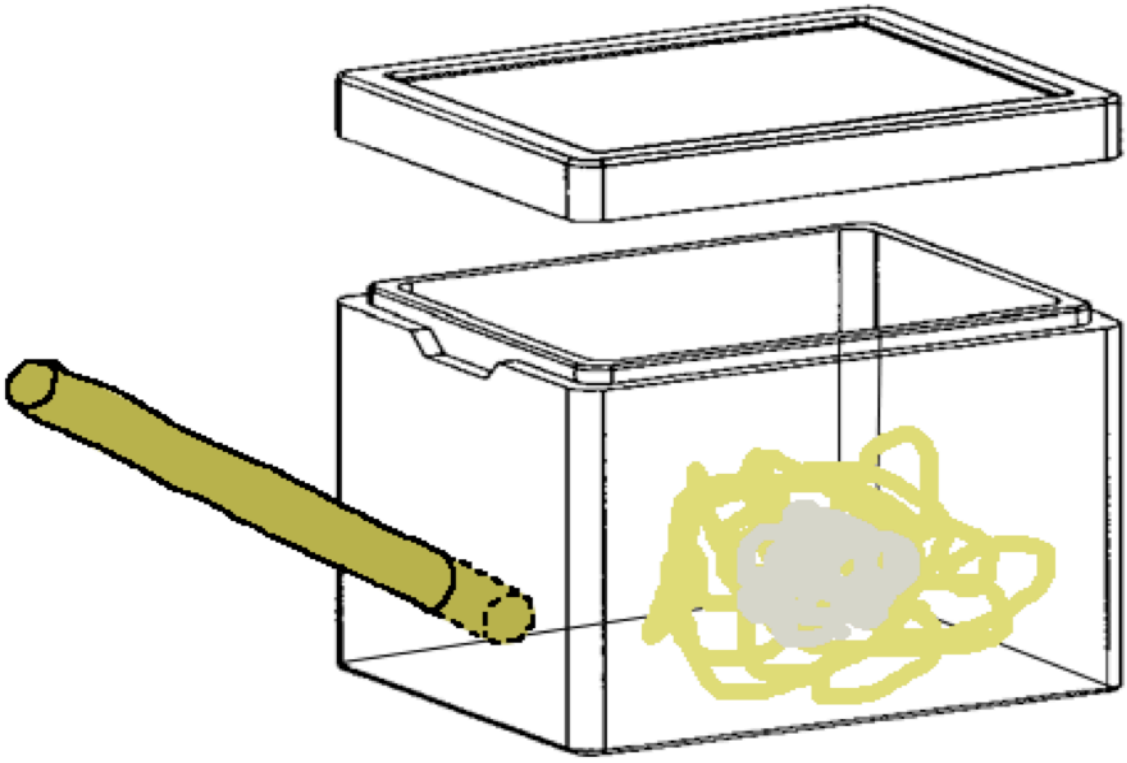

Figure A2.1: Diagram of nest box construction

Appendix 3

Table A3.1: AICc table for the binomial regression models using the proportion of full-melanics (binary format) as the response variable. Sunshine hours are included as a predictor in the model with maximum and minimum temperatures. The data from Twizel were excluded from these models, as none for sunshine hours were available from this site. The global model includes all other predictors.

Model Predictor Variable(s)	K	AICc	Δ AICc	AICc Weight	Cumulative Weight	Log Likelihood	McFaddens Pseudo R^2
Global Model	6	112.60	0.00	0.51	0.51	-50.01	0.183
Humidity + Rainfall	3	112.60	0.22	0.45	0.96	-53.33	0.128
Proportion of *B. hortorum*	2	117.76	5.15	0.04	1.00	-56.84	0.071
Null Model	1	124.41	11.81	0.00	1.00	-61.19	NA
Minimum + Maximum + Sunshine	3	127.40	14.80	0.00	1.00	-60.62	0.009

Table A3.2: AICc table for the regression models using the individual percentage melanic score as the response variable. Sunshine hours are included as a predictor in the model with maximum and minimum temperatures. The data from Twizel were excluded from these models, as none for sunshine hours were available from this site. The global model includes all other predictors.

Model Predictor Variable(s)	K	AICc	Δ AICc	AICc Weight	Cumulative Weight	Log Likelihood	Adjusted R^2
Global Model	6	198.59	0.00	1.00	1.00	-91.91	0.210
Humidity + Rainfall	2	216.42	17.83	0.00	1.00	-104.08	0.088
Proportion of *B. hortorum*	3	221.34	22.75	0.00	1.00	-107.59	0.052
Minimum + Maximum + Sunshine	3	227.54	28.95	0.00	1.00	-108.57	0.027
Null Model	1	228.39	29.81	0.00	1.00	-112.16	NA